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Am J Clin Nutr 2005 81: 330S-335S.
Unlike mathematicians, nutrition researchers often do their best work in middle age. This was true of Clive West, who died on 27 August 2004 in Bennekom, Netherlands. Clive was a world expert on micronutrient malnutrition in developing countries and received international recognition for his research and teaching in this field.

West was born in Griffith, New South Wales, Australia, and studied biochemistry at the University of Sydney. In 1966 he received a PhD from the University of New England in Armidale, New South Wales, for work on the metabolism of free fatty acids in sheep. The same university awarded him a DSc in 1991.

After 3 y of working on animal nutritional biochemistry at the Unilever Research Laboratory in Bedford (United Kingdom), he was appointed Research Fellow at the Australian National University in Canberra in 1968. Here he carried out animal experiments on cholesterol metabolism and also took an interest in fatty acids and cancer. An article on lipoprotein separation that he coauthored with Redgrave and Roberts became a classic. In 1976 Clive moved to Africa, where he became Senior Lecturer in Biochemistry at Ahmadu Bello University in Zaria, Nigeria. Here he saw the effect of micronutrient deficiencies in children and conducted research on blindness caused by vitamin A deficiency.

In 1979 he moved to the Department of Human Nutrition at Wageningen Agricultural University in the Netherlands. His enterprising and enthusiastic personality contributed greatly to building up a fledgling department. Clive studied diet and cholesterol in rabbits but also extended these studies to epidemiology, where he made an important contribution. Short-term feeding trials had shown that low-fat, high-carbohydrate diets depressed serum HDL-cholesterol concentrations and elevated triacylglycerol concentrations, but it was uncertain whether these effects were permanent. Careful studies by West and coworkers of diet and serum lipoproteins in populations across the world showed that the effects of carbohydrates on serum lipids were indeed permanent. It would take another 15 y for these observations to make their way into nutrition policy, but they have undoubtedly contributed to the recent rethinking of optimal carbohydrate intakes.

West maintained his interest in diet and cancer, but funding for a study on diet and atrophic gastritis as a model for gastric cancer proved hard to get. He would later say that this setback was a blessing in disguise, because it led him to return to studying the micronutrient problems that he had first seen in Africa. The group in Wageningen, where Clive worked, was experienced at conducting controlled dietary trials. West adapted this experience to the conduct of large-scale intervention trials on micronutrient deficiencies (particularly of vitamin A, zinc, and iodine), first in Indonesia and then in other tropical countries. That research would “change our understanding about the factors that influence nutrient bioavailability, as well as dietary approaches to improving vitamin A and other micronutrients in the diets of pregnant and lactating mothers and their children,” as quoted from the Kellogg International Nutrition Award that honored him in 2004. One year earlier he had received the Eijkman medal for his research and teaching in tropical nutrition. His best known work is that in which he showed that β-carotene from plants is a poor source of vitamin A. This finding was not readily accepted because, for many years, the consumption of green leafy vegetables had been considered the mainstay for preventing xerophthalmia.

Perhaps the most concrete evidence of the effect of West’s work was when the Institute of Medicine recommended halving the vitamin A value of β-carotene, although Clive thought that halving the value did not go far enough. His other field trials—carried out under sometimes near-impossible circumstances in Tanzania, Ethiopia, Indonesia, and Malawi—showed that vitamin A deficiency is an important factor in the etiology of nutritional anemia and that zinc deficiency is probably one of the most important factors in the etiology of stunting. The size and quality of these trials was remarkable. As he wrote, “There is no reason for the quality of research in developing countries to be lower than that in developed countries. The apparent lack of infrastructure, facilities and trained personnel is no excuse for poor experimental design and lack of quality in data collection.” He also recognized the crucial role of food-composition data in nutrition research, chaired management committees of European Union projects on food composition, and directed or advised training courses on food composition in the Netherlands, South Africa, Chile, Jamaica, and Thailand.

When he returned from a field trip, he would often relay anecdotes about fences that he had climbed, luggage that had to be retrieved, and breakdowns that required improvisation. Clive was prepared to fight for what he believed in, did not knuckle under easily, and had energy that seemed boundless. In addition to his many activities in Wageningen and developing countries, he was a visiting professor at Emory University in Atlanta and

Clive West, PhD, DSc
1939-2004
Professor of Human Nutrition at the Nijmegen University Medical Center in the Netherlands.

Although he knew that he had a serious illness, Clive never let it stop him from doing what he thought was necessary. By now it had become obvious to the world that here was someone who knew about vitamins and minerals, who had a vision of the way forward, and who had the energy and the know-how needed to create new knowledge. He was involved in a major project for developing and testing genetically modified plant foods to combat micronutrient deficiencies, on which he worked with increasing urgency. Regrettably, he would not be allowed to see it through to the end. Nevertheless, at his farewell party 6 wk before his death, he gave a spirited and witty talk and was visibly moved by the announcement of the Professor Clive West Micronutrient Fund (Internet: http://www.westmicronutrient.nl).

Clive West’s untimely death robbed the nutrition field of a scientist with true insight about and a rare vision of ways to prevent micronutrient malnutrition and robbed his many friends, colleagues, and students of a man who always brought a smile to their faces. He was brash, passionate, always fair, and much beloved. I will miss him.

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When to eat and how often?1,2

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The effect of the timing of food intake on metabolism has been the subject of active investigation for >40 y. Indeed, whether it is “better” to eat many small meals a day is one of the questions most frequently posed by the lay public. Comparing the potential benefits of nibbling and of gorging has been the focus of much animal and human research, but no clear consensus has emerged (1–7). Simply put, the question of whether there is a health benefit from the consumption of multiple small meals will ultimately depend on how much energy is consumed, as opposed to how often or how regularly one eats. This possibility raises 2 questions. First, is it easier to overeat under a regimen of frequent, irregular meals? Second, how does the pattern of meal consumption affect metabolic health? These critical issues begin to be addressed in an article by Farshchi et al (8) in this issue of the Journal. Those authors investigated whether an irregular meal pattern (ie, irregular eating frequency) led to changes in daily energy intake and negative metabolic effects that could contribute to obesity and insulin resistance.

In their study, 10 obese but otherwise healthy women participated in 2 free-living dietary phases, each 2 wk in duration, in a randomized crossover design. In one phase of the study, subjects were asked to consume their usual diet on 6 occasions/d, and, in the other phase, subjects were asked to consume their usual diet in a prescribed, yet chaotic fashion (ie, 7, 4, 9, 3, 5, 8, 6, 5, 9, 8, 3, 4, 7, and 6 occasions on days 1–14 of the study, respectively). This elegant study design ensured that the average number of prescribed eating occasions per day was the same in the 2 phases. A standardized test meal, consumed at the beginning and end of each phase, was of constant composition at each feeding. As a result, differences in postprandial responses between the 2 phases reflected the influence of chronic changes in the background pattern of food intakes. As shown by Farshchi et al, the regular eating frequency was associated with lower reported ad libitum energy intakes and lower fasting total and LDL-cholesterol concentrations. In addition, they concluded that the irregular eating frequency may have reduced insulin sensitivity because that pattern was associated with a lower thermic effect of food (TEF), a higher peak insulin concentration, and a larger 3-h area under the curve of postprandial insulin concentrations. One practical limitation of the work by Farshchi et al is that each dietary phase was only 14 d long and thus did not result in large effects (eg, an ≈9% greater peak postprandial insulin concentration and an 8% lower TEF). However, it has long been recognized that the TEF provides a satiety signal (9), and, therefore, when TEF is reduced in persons who are eating irregularly, an increase in body weight may result. Of note, the changes in metabolism observed in the obese subjects studied by Farshchi et al were nearly identical to those previously found in lean women by the same investigators with the use of the same study design (10).

The authors’ interpretation of negative metabolic changes resulting from the irregular timing of food intake raises several questions that are extremely important to clinical nutrition today. First, what are the characteristics of persons who eat irregularly, and what proportion of the general population do they make up? The answers to these questions are not yet known, and Farshchi et al (8, 10) did not report the usual eating patterns of their subjects. Second, if irregular eating frequency is indeed prevalent, how much does that irregular frequency contribute to obesity compared with other eating behaviors that may also influence energy intake (11)? Third, is the specific time of day that food is eaten important? With respect to this question, the potential for breakfast food consumption to reduce total daily intake (12) and the capacity for smaller evening meals to aid in weight loss (13) have both drawn renewed interest of late.

A major hurdle to overcome before these 3 important questions can be answered is that of the poor validity of energy intake records, particularly those from overweight persons (14, 15). This problem is illustrated nicely by carefully examining the energy intakes reported by Farshchi et al for obese (8) and lean (10) women: these intakes were approximately the same at baseline (8.37 MJ/d in the lean women and 8.47 MJ/d in the obese women), even though the obese women clearly had higher energy requirements (15, 16). A comparison of those reported intakes with the energy requirements for women of average height (1.65 m) who are in the same body mass index range and who have a low physical activity level (1.4–1.6; see Table 5-30 in reference 16) shows that the average underreporting by the obese women may have been 22–27%. Farshchi et al suggest that the obese women may have consistently underreported their energy intakes in both phases of the study. However, the degree of underreporting is known to increase with energy requirements (15, 17) and, possibly, with eating frequency (7). Thus, the implication is that the effect of an irregular eating frequency on ad libitum energy intake may have been underestimated by Farshchi et al (8, 10), particularly in the obese women, because there was...
a small increase in energy intake with increased eating frequency during the irregular eating protocol. In addition, whereas irregular eating frequency was associated with a significant increase in energy intake in the obese but not the lean women studied earlier, the group differences in reported energy intakes between the 2 eating regimens were similar (0.40 MJ/d in the lean women and 0.34 MJ/d in the obese women). The significance of the findings in one study but not of those in the other may have been due to the fact that the reported energy intake variances differed between the 2 samples, which reflects the difficulty of obtaining consistently valid self-reports of energy intakes. This problem is not by any means unique to the studies of Farshchi et al but instead is found among nearly all studies in which free-living energy intakes are measured (18).

Two last issues raised by Farshchi et al (8) are whether the effects on metabolism of eating regularity are independent of or mediated by energy intake, and, if there are independent effects, what mechanisms contribute to these effects. With respect to insulin resistance, endocrinologists have long known that, when diabetics are hospitalized for observation, they have significant improvements in blood glucose and insulin concentrations—an effect partially caused by the consumption of regular balanced meals (19). Yet the exact mechanism supporting improved insulin response is unknown.

The increase in obesity over the past decade requires a better understanding of meal timing and eating frequency. The study by Farshchi et al raises key issues of body weight and food consumption and, once again, highlights the urgent need to improve the methods of obtaining valid energy intake records. Whether some persons may be more susceptible than others to increasing their energy intake amid the hustle and bustle of today’s lifestyle is key. Future studies directed toward ascertaining the importance of the timing of eating, as compared with other dietary factors, to energy intake and metabolism will aid immensely in the formulation of innovative therapeutic and preventive strategies for weight control and chronic disease.

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Soy protein with or without isoflavones: in search of a cardioprotective mechanism of action1,2

Penny M Kris-Etherton and Sheila G West

In an article published in this issue of the Journal, Vega-López et al (1) report on a carefully controlled feeding study that they conducted to evaluate the independent effects of soy protein or soy-derived isoflavones on plasma antioxidant capacity and biomarkers of oxidative stress. Prevention of lipid oxidation and, specifically, LDL oxidation is thought to be potentially important for the prevention of atherosclerosis and ischemic heart disease (2). Because no one marker of oxidative stress can provide an accurate picture of either antioxidant status or oxidative stress in an organism (3), the authors measured plasma antioxidant concentrations and a variety of markers of oxidative stress. Despite the finding that plasma total antioxidant performance was modestly increased (by 10%) at the end of the soy-protein phases and that the isoflavones studied had in vitro antioxidant properties, the authors reported no significant effects of the soy diets on the biomarkers of oxidative stress measured. These findings, in conjunction with those of clinical studies showing that soy protein has a substantive effect on blood cholesterol concentrations only in persons with markedly elevated blood cholesterol concentrations (4) and results in clinically meaningful reductions in blood pressure mainly in hypertensive persons (5), temper enthusiasm about the role of soy in reducing the risk of cardiovascular disease (CVD).

Oxidative processes are thought to be important in the initiation and progression of atherosclerosis. The oxidative hypothesis of atherosclerosis is based on the oxidative modification of LDL and phospholipids, which leads to foam cell formation and proliferation, which create an inflammatory state. Current thinking is that antioxidants inhibit lipid peroxidation and, thus, protect against CVD (6). Although this has been the focus of much research, it is possible that other oxidative systems are involved that have not been studied or identified. Recently, interesting data have emerged that may provide clarity about the antioxidant hypothesis. Zheng et al (7) have shown that apolipoprotein A-I (apo A-I), the primary protein constituent of HDL, is a target for myeloperoxidase-catalyzed nitration and chlorination in vivo and that the myeloperoxidase-catalyzed oxidation of HDL and apo A-I inhibits cholesterol efflux from macrophages. Interestingly, these authors showed that apo A-I enriched in nitrotyrosine and chlorotyrosine was present in human atherosclerotic lesions. Additional studies may provide mechanistic evidence that fulfills the oxidative hypothesis theory by a novel system involving HDL.

It is also possible that antioxidants may exert beneficial effects on other mechanisms important for heart health. For example, antioxidants may enhance endothelial nitric oxide synthase activity. An increase in endothelial nitric oxide synthase coupled with an increase in nitric oxide production in hypercholesterolemic vessels may enhance endothelial function. Evidence indicating that soy protein improves endothelial function, as measured by flow-mediated dilation of the brachial artery (5, 8, 9), supports this possibility.

Historically, epidemiologic observations of diet and CVD in Japan have linked soy-product consumption with a decreased risk of CVD (10). More recently, the Shanghai Women’s Health Study reported that soy-food consumption was associated with a reduced risk of coronary heart disease, especially nonfatal myocardial infarction, in women (11). However, large-scale randomized controlled trials have not consistently shown a beneficial effect of antioxidant supplements on CVD morbidity and mortality endpoints (12). Although these negative results refute the oxidation hypothesis, it is possible that other antioxidants not tested could be involved.

For example, α-tocopherol does not inhibit many of the oxidation pathways in human atheroma, including those mediated by myeloperoxidase-catalyzed halogenation and nitric oxide–derived oxidants. In addition, α-tocopherol and ascorbate do not affect systemic measures of oxidant stress. Thus, soy protein may exert effects via changes in endothelial health or by affecting other oxidative systems. Clearly, additional studies are needed to resolve this issue.

In summary, the benefits of soy—if any—require further investigation to determine the mechanisms responsible for these effects. Until then, it remains prudent to recommend soy products in a heart healthy diet because of their nutritional value and as a healthy substitute for protein sources that are higher in saturated fat and cholesterol.

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Dietary fats, teas, dairy, and nuts: potential functional foods for weight control?1–3

Marie-Pierre St-Onge

ABSTRACT
Functional foods are similar to conventional foods in appearance, but they have benefits that extend beyond their basic nutritional properties. For example, functional foods have been studied for the prevention of osteoporosis, cancer, and cardiovascular disease. They have yet to be related to the prevention of obesity, although obesity is one of the major health problems today. The inclusion of foods or the replacement of habitual foods with others that may enhance energy expenditure (EE) or improve satiety may be a practical way to maintain a stable body weight or assist in achieving weight loss; such foods may act as functional foods in body weight control. Some foods that might be classified as functional foods for weight control because of their effects on EE and appetite—including medium-chain triacylglycerols, diacylglycerols, tea, milk, and nuts—are reviewed here. Only human studies reporting EE, appetite, or body weight are discussed. When studies of whole food items are unavailable, studies of nutraceuticals, the capsular equivalents of functional foods, are reviewed. To date, dietary fats seem to be most promising and have been the most extensively studied for their effects on body weight control. However, the weight loss observed is small and should be considered mostly as a measure to prevent weight gain. Carefully conducted clinical studies are needed to firmly ascertain the effect of tea, milk, and nuts on body weight maintenance, to assess their potential to assist in weight-loss efforts, and to ascertain dose-response relations and mechanisms of action for the 4 food types examined. Am J Clin Nutr 2005;81:7–15.

KEY WORDS Medium-chain triacylglycerols, diacylglycerols, tea, milk, nuts

INTRODUCTION
Maintenance of a constant body weight requires a balance between energy intake (EI) and energy expenditure (EE), and even a slight imbalance in this energy equilibrium can lead to significant changes in body weight over time and may eventually result in obesity (1). Obesity is one of the major health problems worldwide, and it is a risk factor for several chronic disorders, but there is no functional food for obesity, such as there is for cardiovascular disease (CVD) or cancer. In the Health Professionals Follow-up Study, mean weight change over a 10-y period was 1.8 kg (2). Even small changes in energy balance may lead to such a weight gain, which therefore may be prevented by slight modifications in food intake, such as the inclusion of functional foods for weight management.

Although it is known that dietary restriction and increased physical activity can lead to weight loss, such lifestyle changes may be difficult to implement and maintain—thus, the high rate of recidivism among weight losers (3). Functional foods that effect energy metabolism and fat partitioning may be helpful adjuncts to a dietary approach to body weight control. The current review examines current literature to identify potential functional foods that may be useful in the prevention of weight gain or as adjuncts to weight-loss efforts. It is not within the scope of this review to examine overall diets or individual food components, such as vegetarian diets and fiber or protein, so that only foods that have been studied for their effects on body weight, EE, or satiety (or all 3) are reviewed. Such potential functional foods that may be of interest include medium-chain triacylglycerols (MCTs), diacylglycerols, tea, milk, and nuts.

DIETARY FATS
Medium-chain triacylglycerols
MCTs are those triacylglycerols composed of fatty acids that contain 6–12 carbon atoms. These triacylglycerols differ from long-chain triacylglycerols (LCTs) not only in their chemical composition but also in the manner in which they are absorbed and transported from the gastrointestinal tract to organs. Both MCTs and LCTs are digested to their respective medium- and long-chain fatty acids (MCFAs and LCFAs, respectively) in the gastrointestinal tract. Unlike LCFAs, which are repackaged as LCTs into chylomicrons for transport through the peripheral circulation, MCFAs, because of their shorter chain lengths, do not require chylomicron formation for their absorption and transport (4, 5). As a result, MCFAs travel directly to the liver via the portal circulation; therefore, they bypass peripheral tissues such as adipose tissue, which makes them less susceptible to the actions of hormone-sensitive lipase and to deposition into adipose tissue stores (Figure 1). In fact, MCFAs are mostly oxidized by...
Long-chain fatty acids (LCFAs) are packaged into chylomicrons for their transport to peripheral tissues, whereas medium-chain fatty acids (MCFAs) travel directly to the liver via the portal circulation. As a result, LCFAs are mostly deposited into adipose tissue, whereas MCFAs are mostly oxidized to carbon dioxide in the liver, and small amounts are elongated to LCFAs and incorporated into complex lipids.

The liver for use as a source of energy and thus have been reported to behave more like glucose than like fats (5). The metabolic differences between MCTs and LCTs prompted researchers to examine their effects on EE and body composition. Several reviews discussed the physiologic processing of MCTs (4–6).

Early studies examined the effects of MCTs on EE in humans (7–9). Those investigators mostly reported a greater thermogenic effect of MCTs than of LCTs, but their studies were of a short duration (10). As a result, although it was suggested that the consumption MCTs could lead to an energy imbalance that may assist in weight loss or in the prevention of obesity (7–9), those early studies did not provide evidence of the longer-term effects of MCTs on thermogenesis. Furthermore, the short-term nature of the studies precluded the possibility of examining body-composition changes that would theoretically result from the energy imbalance.

In the School of Dietetics and Human Nutrition at McGill University, we recently used a 4-wk crossover feeding experiment to compare the effects of MCT and LCT consumption on EE and body composition in both men (11) and women (12). In both studies, subjects were fed controlled diets that were designed to maintain body weight and that differed only in the type of added dietary fat: MCTs or LCTs. Diets were fed for 4 wk and were separated by a 4-wk washout period. EE and body composition, measured by magnetic resonance imaging, were assessed at baseline and the endpoint of each experimental phase. As a result, we could determine whether the greater effects of MCTs than of LCTs on thermogenesis were maintained over a 4-wk period and whether any increase in EE with MCT consumption over that seen with LCT consumption would result in significant changes in body composition, regardless of the isoenergetic content of the diets. We found that EE increased with the consumption of MCTs more than it did with that of LCTs at baseline and at the endpoint in men and women; however, the extent to which thermogenesis was increased varied slightly. In women, differences in daily EE, extrapolated in the same manner, equated to 263.3 kJ (63 kcal) at baseline and 179.7 kJ (43 kcal) at the endpoint (11). Although body weights did not change significantly in women, variations in body weight in both men and women could be explained by the differences observed between EE with MCT consumption and that with LCT consumption. Moreover, total adipose tissue, subcutaneous adipose tissue, and upper-body adipose tissue stores in men decreased significantly with MCT consumption but not with LCT consumption (11).

Furthermore, data suggest that MCT consumption increases satiety more than does LCT consumption (10). In a subgroup of men, we tested the potential effect of MCT consumption on food intake at a subsequent meal (12). After consuming the fixed-intake breakfast containing either MCTs or LCTs, men were instructed to eat as much as they liked of a different, ad libitum lunch that did not contain the experimental fats. Although the sample was small \( n = 5 \), there was a trend toward lower EI at the lunch after the MCT-containing breakfast than at the lunch after the LCT-containing breakfast. The slightly lower EI \([-925 kJ (221 kcal)]\) was due to significantly lower fat intake \(-12.4 g\) at the lunch after the MCT-containing breakfast than at the lunch after the LCT-containing breakfast (12).

These results add to the body of literature examining the effects of MCT consumption on EE and further confirm the potential of MCTs to act as dietary adjuncts for improved body weight maintenance or even, possibly, weight loss. However, weight-loss studies are needed to confirm this latter role of MCTs. Found in the form of liquid oil extracted from coconut oil, MCTs could easily be incorporated into the North American diet as a replacement for other LCT-rich vegetable oils. A study examining the acceptability (ie, appeal to the consumer) of different food items made with an MCT oil found that the drop cookies, muffins, and quick loaf breads were acceptable, but the rolled cookies were not (13). In clinical studies in which one-half of the total fat intake was from an MCT oil, it was found that incorporation of an MCT-rich oil into cakes and cookies produced acceptable products. Moreover, subjects had no complaints about the taste of the oil or of the mashed potatoes, pasta, or desserts in which it was incorporated (M-P St-Onge, personal observations, 1999–2001). One must keep in mind, however, that baked goods are typically high in energy, and these foods should be consumed sparingly even if the replacement of LCTs by MCTs contributes to the enhancement of EE and satiety. An oil containing only MCT could be incorporated into foods as part of a salad dressing, but it may not be appropriate for frying because of its low smoke point. One possible way to circumvent this problem would be to combine an MCT oil with other vegetable oils having higher smoke points, such as canola oil or safflower oil, which would then make the MCT oil more suitable for stir-frying and baking.

There are, however, some concerns regarding the effects of MCT consumption on plasma lipid concentrations (14). In fact, a recent study (15) found an intake of 70 g MCT oil/d for 21 d increased total cholesterol, LDL-cholesterol, triacylglycerol, and glucose concentrations by 11%, 12%, 22%, and 4%, respectively, relative to an equivalent intake of high-oleic sunflower oil. There is thus some concern regarding the cardiovascular effects of MCTs. One possible way to prevent adverse cardiovascular effects would be to combine plant sterols, which have showed benefits for cholesterol concentrations (16), with an MCT oil. When this combination is consumed, total and LDL-cholesterol

![Figure 1](image.png)

**FIGURE 1.** Long- and medium-chain triacylglycerols are digested to their respective fatty acids in the gastrointestinal tract. Long-chain fatty acids (LCFAs) are packaged into chylomicrons for their transport to peripheral tissues, whereas medium-chain fatty acids (MCFAs) travel directly to the liver via the portal circulation. As a result, LCFAs are mostly deposited into adipose tissue, whereas MCFAs are mostly oxidized to carbon dioxide in the liver, and small amounts are elongated to LCFAs and incorporated into complex lipids.
amount of body weight and fat mass, but the changes were greater the level of the L4–L5 vertebrae. Both groups lost a significant sorptiometry and single-slice computed tomography scanning at composition was assessed by whole-body dual-energy X-ray ab-

cluded in this randomized, parallel-arm experiment had a waist circumference

visceral and subcutaneous adipose tissue at the level of the um-

acrilicus were significantly greater with diacylglycerol than with triacylglycerol-supplemented group was 1.1 kg. The reductions in body mass index (BMI; in kg/m²), waist circumference, and visceral and subcutaneous adipose tissue at the level of the umbilicus were significantly greater with diacylglycerol than with triacylglycerol supplementation. The authors concluded that diacylglycerol supplementation suppresses body weight and regional fat deposition. However, in both groups, total fat consumption, including the test or control fat, did not meet the 50 g/d requirement and was assessed to be 43 g/d. This lack of compliance with the study protocol may have been partly responsible for the changes observed in body composition. Furthermore, energy and fat intakes for each group were not provided.

A weight-loss study including overweight and obese men and women found that body weight and fat mass decreased to a greater extent in subjects consuming diacylglycerols than in those consuming triacylglycerols (24). All men and women included in this randomized, parallel-arm experiment had a waist circumference > 90 cm (men) or > 87 cm (women). Subjects were asked to reduce their caloric intakes by 2090–3344 kJ/d (500–800 kcal/d) for a period of 24 wk, during which they incorporated foods containing diacylglycerols or triacylglycerols. Foods provided 16–45 g/d of either diacylglycerols or triacylglycerols, or 15% of the subjects’ energy requirements. Body composition was assessed by whole-body dual-energy X-ray absorptiometry and single-slice computed tomography scanning at the level of the L4–L5 vertebrae. Both groups lost a significant amount of body weight and fat mass, but the changes were greater in subjects who were supplementing their diets with diacylglycerol-containing foods than in those who were supplementing their diets with triacylglycerol-containing foods (body weight change: −3.6% and −2.5%, respectively). The percentage change in intraabdomi-

Although these previous studies did not examine the potential mechanism leading to greater effects of diacylglycerols than of triacylglycerols on loss of fat mass, it seems likely that the effects could be due to greater EE, fat oxidation, or reductions in appetite with diacylglycerol than with triacylglycerol consumption rather than to differences in the energy contents of diacylglycerol and triacylglycerol. Diacylglycerol and triacylglycerol have been shown to have similar energy values [37 kJ/g (9 kcal/g)] (25). In addition, a recent study showed that, when 12% of total daily EI was provided by diacylglycerol in foods, fat oxidation was greater than that when EI was provided by triacylglycerol in foods (23). EE did not differ significantly between the groups, but subjects reported being less hungry (area under the curve score: 281 and 472 mm·h, respectively) after diacylglycerol consumption than after triacylglycerol consumption. This study provides some information on the reasons for weight loss with diacylglycerol consumption, but much remains to be established. Furthermore, the previous feeding experiments (19, 24) were parallel-arm supplementation studies, and therefore the exact food intake of subjects could not be established with certainty, which may have worked to confound the results obtained.

Diacylglycerols occur naturally in small concentrations in several edible oils; the 9.5% concentration in cottonseed oil is among the highest (26). A diacylglycerol-rich cooking oil has been produced that contains >80% diacylglycerols and that looks and tastes like a conventional oil (26). Such an oil could therefore be incorporated into foods or consumed as a salad dressing without imparting a distinct flavor to the food.

Studies examining the effect of changes in dietary fat type (11, 12, 19, 24) seem to indicate that this slight dietary modification may be beneficial for body weight control and weight loss. In fact, the Japanese government has approved diacylglycerol as a food for specific health use to control postmeal blood lipids and body fat (26). However, the magnitude of this effect is small when observed in a controlled setting, and therefore dietary fats may be most helpful in the prevention of weight gain when used alone or in the enhancement of weight loss when incorporated in a more rigorous weight-loss plan.

**BEVERAGES**

**Tea**

Tea is the beverage with the greatest consumption worldwide (27). There are 3 categories of tea—black, green, and oolong—and the consumption of black tea accounts for 80% of total tea intake (27). Black tea leaves are fermented and contain mostly theaflavins and thearubigins as active components (27). Green tea is a nonoxidized, nonfermented tea, which contains polyphenolic compounds such as epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate (EGCG), whereas oolong tea is partially oxidized and contains a considerable amount of polyphenols (27). Tea polyphenols have been found to be powerful antioxidants that may reduce LDL oxidation and the formation of oxidized DNA metabolites, thus contributing to lower risks of CVD and cancer (28).
Few studies have carefully examined the effects of tea consumption on body weight or EE. In fact, only 2 studies have examined the effect of tea consumption, as a beverage, on EE (29, 30). Rumpler et al. (29) tested whether oolong tea increased EE or modulated substrate oxidation rates more than did control beverages. Four different beverages were consumed 5 times/day for 3 d each: full-strength oolong tea (3 g tea leaves in 300 mL water), half-strength oolong tea (1.5 g tea leaves in 300 mL water), water, and water + caffeine (caffeine content equivalent to that of full-strength tea). Each serving of full-strength oolong tea contained 48.7 mg EGCG and 53.7 mg caffeine, for a total intake of 244 mg EGCG and 270 mg caffeine. Servings were consumed every 1.5 h from 0830 to 1430 as part of a controlled diet providing 115% of energy requirements during the initial 2 d and 100% of energy requirements on the 3rd day. Twenty-four-hour EE with full-strength tea and water + caffeine consumption was significantly greater than that with water alone. The increase in EE with consumption of full-strength tea and for water + caffeine was, respectively, 2.9% [281 kJ (67 kcal)] and 3.4% [331 kJ (79 kcal)] greater than that for water. Fat oxidation increased by 12% relative to baseline for full-strength tea and by 8% for water + caffeine. However, only the consumption of full-strength tea resulted in significantly greater fat oxidation (13.1%) than did the consumption of water. The authors concluded that oolong tea stimulated EE and fat oxidation in normal-weight males and could have some beneficial effects on a person’s ability to maintain lower body fat. However, they also cautioned that weight maintenance would be facilitated only if the effects of tea consumption on EE and fat oxidation were sustainable and if no dietary compensation occurred to offset the slight energy imbalance.

Another recent study examined the effects of oolong tea and green tea consumption on fasting EE (30). Eleven healthy normal-weight women were tested after drinking water and again after drinking oolong and green tea in random order. Oolong tea was prepared by brewing 15 g tea leaves in 300 mL water, and each serving contained 77 mg caffeine and 81 mg EGCG. Green tea was prepared by dissolving 5 g powdered green tea in 300 mL water. Each serving of green tea contained 161 mg caffeine and 156 mg EGCG. EE was measured by using the Douglas bag method both at baseline and for 2 h after beverage consumption. Resting EE was similar in the 2 groups before consumption of the different beverages and remained low after water and green tea consumption but increased significantly after oolong tea consumption. The cumulative increase in EE over resting EE after the consumption of oolong tea, green tea, and water was 110.7 (26.5 kcal), 49.5 (11.8 kcal), and 11.2 kJ (2.7 kcal), respectively, over the 2-h measuring period. Respiratory quotients did not differ between the 3 treatments. The authors concluded that, because oolong tea had less caffeine and EGCG than did green tea, the rise in EE must be due to the presence in oolong tea of more polyphenolized polyphenols than are found in green tea. However, the measurement period in this study was very short, and it is not known whether the increased EE would extend over the full postprandial period—typically 6–7 h—or whether increases would be observed on subsequent tea-drinking occasions.

Another study that examined the effects of tea on thermogenesis (31) used tea as a nutraceutical rather than a functional food. Nutraceuticals are components that are isolated or purified from a food or beverage, that have been shown to have health benefits or reduce the risk of chronic disease, and that are usually found in capsular or nonfood format (32). Healthy young men underwent three 24-h testing periods in a metabolic chamber to examine whether 2 capsules of a green tea extract containing 50 mg caffeine and 90 mg EGCG, taken 3 times/d, stimulated thermogenesis to a greater extent than did caffeine alone or placebo. Twenty-four-hour EE with green tea extract treatment was greater than that with caffeine and placebo—9867 kJ (2360.5 kcal) and 9599 (2296.4 kcal) and 9538 kJ (2281.8 kcal), respectively—which corresponds to an increase of 2.8% and 3.5% [268 kJ (64.1 kcal) and 329 kJ (78.7 kcal), respectively] over the EE with caffeine and placebo, respectively. The 24-h respiratory quotient with green tea extract treatment was lower than that with caffeine and placebo—0.852 compared with 0.873 and 0.881, respectively—which is indicative of greater fat oxidation with green tea extract treatment. It was concluded that oral administration of a green tea extract stimulated thermogenesis and fat oxidation and, therefore, that green tea extract has the potential to influence body weight and body composition. However, the short-term nature of the study prevented the direct observation of the effect of green tea extract intake on body composition.

Dulloo et al. (33) also confirmed the mechanism of action of the green tea extract in an in vitro experiment. This experiment showed that the green tea extract significantly increased the rate of intrascapular brown adipose tissue oxygen uptake, but caffeine alone did not. The authors concluded that the green tea extract was a more effective potentiator of sympathetically mediated thermogenesis than was caffeine alone. In humans, this green tea extract was also found to reduce body weight by 4.6% and waist circumference by 4.5% when 2 capsules were taken twice daily for 12 wk as part of a regular, self-selected diet (34). Each capsule contained 375 mg catechins, including 270 mg EGCG. However, this study did not include a control group or a report of the statistical procedures, and therefore clear conclusions cannot be drawn from these results.

To date, no study has established the potential of tea as a functional food for weight maintenance. The only studies that examined tea as a functional food found modest effects on EE and were of very short duration (29, 30). Whether these slight increases in EE and fat oxidation persist over a long period remains to be established. Therefore, more research is necessary to ascertain whether tea can be of assistance in better weight maintenance or in weight-loss programs and whether its effects are more than those exerted by its caffeine content. Moreover, differences between types of tea with respect to their effect on EE should be explored further. As yet, the quantity of tea that must be consumed to obtain an effect on body weight has not been established. A study in rats in which intraperitoneal injections of 100 mg EGCG/kg body weight for 7 d resulted in losses in body weight and fat mass found that plasma concentrations of EGCG after injection were 24, 2, 4, 1, and 1 μmol/L at 0.5, 1, 2, 5, and 24 h, respectively (35). A concentration of 1 μmol/L would be similar to that in a 70-kg person 1 h after drinking 6–12 200-mL servings of green tea.

**Milk**

Although a meta-analysis of calcium consumption and weight loss has not shown that calcium consumption is linked to greater loss of body weight (36), there is increasing evidence that dairy calcium may play a role in body weight regulation (37, 38). Recently, Heaney (39) reported the effects of calcium consumption on body weight and the rate of body weight change in a
longitudinal cohort of women. This study showed that predicted BMI decreased with an increased ratio of calcium to protein, so that a ratio of 10 predicted a BMI of 22.5, whereas a ratio of 20 predicted a BMI of 19.3. Calcium:protein of 9, corresponding to approximately the 25th percentile of the currently recommended intakes, predicted weight gain at midlife is 0.425 kg/y. The observed rate of weight gain was, however, 1 kg/y. If calcium: protein was 20, representative of the current recommendations, the predicted weight change would be −0.011 kg/y, and only 3.7% of women would be predicted to gain 1 kg/y. The author suggested that the prevalence of obesity could be decreased by 60–80% in women if their calcium intakes were at recommended amounts (39). These observations corroborate those of others (40, 41), who reported associations between calcium intakes and body weight. From the third National Health and Nutrition Examination Survey, it was found that the risk of obesity was 85% lower in those in the highest quartile of calcium intakes, after adjustment for age, sex, race, and EIIs, than in those in the lowest quartile (41). Similarly, in the Coronary Artery Risk Development in Young Adults Study, dairy consumption was inversely associated with the prevalence of obesity (40), and Davies et al (42) reported that the odds ratio for overweight with calcium:protein below the median (≈12 mg/g) was 2.25.

Moreover, calcium intakes recently were reported to be negatively associated with fat mass in the Quebec Family Study (43). The authors of this study found that women who consumed <600 mg calcium/d had greater body weight (82.3 kg compared with 69.8 and 65.0 kg for those consuming 600–1000 mg and >1000 mg calcium/d, respectively), BMI (31.8 compared with 27.0 and 25.0, respectively), percentage fat mass (37.3% compared with 31.3% and 28.9%, respectively), absolute fat mass, waist circumference, and abdominal adipose tissue than did women who consumed greater amounts of calcium, even after adjustments for EI, percentage of energy consumed as fat, dietary protein intake, socioeconomic status, and age.

A potential mechanism of action of milk in the promotion of weight loss has already been put forth (44). It is proposed that intracellular calcium plays a role in adipocyte metabolism and that its concentrations are modulated by calcitrophic hormones. An increase in calcium intake in foods would down-regulate 1,25-dihydroxyvitamin D, which would result in a decrease in the absorption of calcium into adipocytes and pancreatic islet cells (Figure 2). Within the adipocyte, intracellular calcium increases fatty acid synthase transcription and inhibits lipolysis. Within the pancreas, intracellular calcium stimulates insulin release, which further acts to inhibit lipolysis and stimulate fatty acid synthase transcription. Therefore, any reduction in intracellular calcium would lead to a reduction in lipogenesis and the stimulation of lipolysis (44). This is supported by recent data showing that acute dietary calcium intake was correlated positively with 24-h fat oxidation and negatively with the 24-h respiratory quotient (45).

However, in a controlled feeding experiment in which subjects were given diets containing 500 or 1400 mg dairy calcium/d in a crossover design for 7 d each, EE and substrate oxidation did not differ significantly between diets (46). However, other compounds within dairy products may act in concert with dietary calcium to produce antiobesity effects. Such compounds that have been proposed are whey proteins (44), conjugated linoleic acid (47), and branched-chain amino acids (48). However, a recent study showed no effect of various conjugated linoleic acid isomers on body weight loss per a period of 18 wk (49).

Cross-sectional studies (40, 41, 43) have thus found a relation between milk or calcium consumption and body weight. Such cross-sectional studies, however, do not establish causal relations, and, therefore, whether the relation between milk consumption and body weight is due to other characteristics of milk consumers is not known. For example, adults who do not eat fast food drink more milk than do those who do eat fast food (50). In addition, milk may be replacing other, more energy-dense beverages in the diet. This, however, is debatable, because some find lower milk intakes in those who consume more soft drinks (51) and others find that soft drink intake is not associated with lower calcium intakes in children and adolescents (52). Furthermore, results have been ambiguous in clinical studies: some showed no effect of calcium (53, 54), and another showed a beneficial effect (55) on weight loss. One randomized clinical study recently examined the effects on bone turnover of protein-rich weight-loss diets that are high in dairy protein and calcium and in mixed protein sources (53). Diets were not strictly controlled, but each subject was given instructions on dietary requirements and was provided with a digital kitchen scale and foods to cover 60% of their total energy prescription. The entire study consisted of 2 phases, an energy-restriction phase of 12 wk and a subsequent energy-balance phase of 4 wk. The dairy protein diet contained 2371 mg calcium, compared with 509 mg calcium for the mixed protein diet, and 62% of its total protein content was from dairy products. Overall body weight loss was 10% during the energy-restriction phase, irrespective of dietary treatment. As planned, there was no weight loss during the energy-balance phase. Although the primary endpoint of interest in this study was not weight loss per se, these results show that, as part of a weight-loss diet, high dairy protein and calcium consumption does not lead to...
greater weight loss than does a diet consisting of mixed protein sources and <600 mg calcium/d.

Similarly, in a study examining the effects of calcium supplementation in limiting bone loss during weight loss (54), there was no effect of calcium on body weight and fat mass loss. Obese postmenopausal and premenopausal women were randomly allocated to a weight-loss diet with a 2100 kJ/d (500 kcal/d) energy deficit with or without 1000 mg elemental calcium/d for 25 wk. Calcium supplements were provided as 2 pills to be taken at breakfast and dinner. The control group received placebo pills. Weight loss did not differ significantly between groups—6.2 and 7.0 kg for the placebo and the calcium supplement group, respectively. Accordingly, fat-mass loss also did not differ significantly between placebo and calcium groups—4.5 and 5.5 kg, respectively. However, this study was not powered to detect changes in body weight and fat mass between groups, and a post hoc power analysis showed that 500 subjects/group would have been necessary to detect a 0.8-kg difference in body weight, and 265 subjects/group would have been required to detect a 1.0-kg difference in fat mass between groups, with 80% power and 95% CIs. The authors thus proposed that the direction of change observed in this study suggests that, over longer periods and with an adequate number of subjects, calcium may have an effect on body weight loss. Moreover, this study provided calcium in its elemental form, and it may be that calcium from dairy products has a greater effect than does elemental calcium because of other components present in dairy foods. However, the results obtained by Bowen et al (53) suggest that this may not be the case.

A recent clinical trial aimed to ascertain whether dairy or elemental calcium supplementation enhanced weight loss in obese men and women (55). Subjects were examined after a 2-wk lead-in period and were then randomly assigned to 1 of 3 groups: group 1 consisted of control subjects who were restricting their caloric intake by 2100 kJ/d (500 kcal/d) and consuming 0–1 serving dairy products/d while taking a 400–500 mg calcium supplement and a placebo pill (low-dairy group); group 2 received the same dietary prescription as group 1, but the placebo pill was replaced by 800 mg calcium carbonate (high-calcium group); and group 3 had the same dietary prescription as group 1 but consumed 3 servings dairy products/d (high-dairy group). Diets were followed for 24 wk. All subjects initially consumed <1 serving dairy products/d. Of the 41 subjects enrolled, 32 completed the study; the data reported include completers only. At the end of the weight-loss period, subjects in the low-dairy, high-calcium, and high-dairy groups lost 6.4%, 8.6%, and 10.9% of body weight, respectively. Fat-mass loss followed the same trend: the low-dairy, high-calcium, and high-dairy groups lost 8.1%, 11.6%, and 14.1%, respectively, of total fat mass. Fat loss from the abdominal region represented 19% of the total fat loss in subjects in the low-dairy group and 50.1% and 66.2% for those in the high-calcium and high-dairy groups, respectively. These data show that calcium, particularly that from dairy products, can enhance weight loss in obese persons. However, only data from those who completed the study were analyzed, and thus results may not represent all subjects. Furthermore, it is not known whether similar results would be obtained in persons who regularly consume larger amounts of dairy products.

A potential mechanism has been postulated to explain a possible role of calcium in body weight control, and yet it remains unclear whether there is a weight-loss effect of calcium. Only one clinical study was specifically conducted to examine the role of calcium as part of a weight-loss program, but data for all of the subjects initially enrolled in the study were not reported (55). Other clinical studies were post hoc analyses and were not specifically designed to test the hypothesis that calcium may play a role in body weight regulation (53, 54). More studies are therefore needed to draw definitive conclusions about the effects of calcium and dairy products on weight management. There is also some concern regarding a possible link between milk consumption and prostate cancer. A recent meta-analysis found an odds ratio of 1.68 for prostate cancer in subjects with high milk consumption when examining 11 case-control studies (56). However, other longitudinal studies have found either no association (57) or a weak positive association (58) between higher milk consumption and prostate cancer (odds ratio: 1.34).

NUTS

Nuts vary widely in caloric content and fat composition and have often been excluded from diets because of their high fat content (59). As a result, some concern existed with respect to the potential effects on body weight of incorporating nuts in the diet. However, many of the supplementation studies that have examined the effects of nuts on lipid profiles have not found negative effects on body weight (60–68), but they did show that nuts—whether almonds (61, 65, 68, 69), walnuts (60, 67, 68), pecans (63), pistachios (66), or peanuts (62, 64)—improve plasma lipid profiles and can have a beneficial effect on CVD risk. Because very few studies have specifically examined the effects of nuts on body weight (70, 71), those studies that examined the effects of nut consumption on plasma lipids and also reported body weight at baseline and endpoint (60–69) will be reviewed here. These studies were not designed to produce weight loss.

Studies examining the effect of almonds on plasma cholesterol concentrations have also reported body weight changes during the experimental and control feeding periods (61, 65, 69). In the study of Spiller et al (61), male and female subjects were examined after a 2-wk baseline period of no intervention and again after 9 wk of supplementation with 100 g almonds/d (2424.4 kJ/d (580 kcal/d)); half of the almonds were whole blanched almonds, and the other half were ground almonds. Subjects were also provided with almond oil to replace other, normally used cooking fats, and they were asked to eliminate margarine, butter, vegetable oils, mayonnaise, most meats, shellfish, whole-fat dairy, high-fat bakery products, potato chips, ice cream, avocado, and all other nuts from their diet. Total fat intake increased from 67 to 90 g/d, and protein intake increased from 88 to 103 g/d, whereas carbohydrate intake decreased by 33 g/d. Although total EI, assessed from 3-d food records at baseline and at weeks 4 and 8 of the intervention period, increased by 338.6 kJ/d (81 kcal/d) over the study period, body weights did not change (74.9 kg at baseline and 74.3 kg at week 9). On the basis of the difference in daily EIs, a theoretical weight gain of 0.66 kg would have been expected over the 9-wk period. Moreover, if the subjects had not partially compensated for the added calories provided by the almonds, a weight gain of 10 kg would have been expected.

More recently, Jenkins et al (65) studied 3 supplements in a randomized crossover design: control muffin, full-dose almond, and half-dose muffin + half-dose almond. The subjects, who were hyperlipidemic men and women, consumed self-selected National Cholesterol Education Program Step 2 diets and were counseled to maintain a stable body weight. Depending on
each subject’s energy requirements, full supplement doses provided 1200 (287 kcal/d), 1797.4 (430 kcal/d), and 2399.3 kJ/d (574 kcal/d) for total energy requirements of 6688 (1600 kcal/d), 6688–10 032 (1600–2400 kcal/d), and >10 032 kJ/d (>2400 kcal/d), respectively. Authors found a dose effect of almonds on CVD risk factors but no change in body weights over the 1-mo supplementation periods. In this study, the lack of body weight change with all supplements may have been due to good compliance with counseled weight-management strategies and not necessarily to a satiating effect of the almonds. Nevertheless, food records during the supplementation periods indicated an energy intake during the full-dose muffin phase that was 560.1 kJ/d (134 kcal/d) more than that during the full-dose muffin phase. This greater caloric intake should theoretically have led to a weight gain during the full-dose muffin phase that was 0.5 kg more than the gain during the full-dose muffin phase. Therefore, assuming consistent reporting of dietary intakes during all phases of this study, there may be some unabsorbable energy in almonds that negated the energy imbalance caused by the greater intakes.

Another supplementation study, however, found slight but significant body weight gains when men and premenopausal women supplemented their diets with 100 g almonds/d for 4 wk (69). This slight weight gain of 0.9 kg for men and 0.3 kg for women occurred despite recommendations to reduce EIs by an amount equivalent to that provided by the almonds: \( \approx 2424.4 \text{ kJ/d (580 kcal/d)} \).

One study examined the effect of walnut consumption on plasma lipid concentrations in hypercholesterolemic men and women (60). Two different diets were tested in a randomized crossover design for 6 mo each. During both phases, subjects were instructed to consume a Mediterranean diet of prescribed energy content, which emphasized vegetable products and fish and limited red meat and eggs. For the control phase, olive oil was advised for cooking, and nuts were not allowed. During the walnut phase, subjects consumed 41–56 g walnuts/d in partial replacement of the olive oil and other fatty foods in the control diet. Despite a trend toward greater EIs during the walnut phase than during the control phase [\( x \pm SD: 7624.3 \pm 744 \text{ kJ/d (1824 } \pm 178 \text{ kcal/d)} \) and 7402.8 \pm 635.4 \text{ kJ/d (1771 } \pm 152 \text{ kcal/d)}, respectively; \( P = 0.11 \)], body weights remained the same during both phases (69.9 and 70.1 kg for walnut and control phases, respectively). The difference in EIs between diets theoretically should have led to a weight gain of 1.2 kg over the 6-mo period.

Another study examined the effect of walnut consumption on plasma lipid concentrations by feeding a habitual diet and a low-fat, free-living diet (67). Men and postmenopausal women consumed their habitual diets for 4 wk and then supplemented those diets with 48 g walnuts/d for 6 wk; they then followed a low-fat (20% of energy) diet for 6 wk and, finally, a low-fat + walnut diet for 6 wk. The walnut dose provided 1570 kJ/d (375.6 kcal/d). EIs during the walnut phases exceeded those during the walnut-free phases by 1651 kJ/d (395 kcal/d) for the habitual diet and 1514 kJ/d (362.2 kcal/d) for the low-fat diet. Regardless of the additional EIs during the walnut supplementation phases, body weights did not increase during the walnut phases.

Similar observations were made when subjects consumed self-selected 8-wk diets with 68 g pecans/d or no nuts (63). Pecan supplementation provided an extra 1918.6 kJ/d (459 kcal/d) and 44 g fat/d. As was observed with walnuts (67), neither body weights nor BMI changed during the supplementation period, despite an increase in EIs and fat intakes.

In a study of pistachio nuts, men and women maintained their regular diets for 3 wk and replaced 20% of their energy intakes with pistachio nuts for the subsequent 3 wk (66). As assessed by food records, subjects consumed the same number of calories and macronutrients during the 2 phases. As expected, there was no significant change in body weight during the study, which showed good compliance with the dietary replacement protocol. Although of short duration, this study shows that subjects can successfully substitute pistachio nuts for other foods in a regular, free-living diet without increasing their body weights.

All of the above-mentioned studies except that of Lovejoy et al (69) found no weight gain with nut consumption, despite increases in EIs. If we assume adequate reporting of EIs, this absence of body weight gain may have been due to some degree of malabsorption of energy in nuts or to an increase in EE with nut consumption. The absence of body weight gain with nut supplementation led to studies that examined the effects of nut consumption on body weight and energy balance (70, 71).

The study by Fraser et al (71) tested the effect of consuming 1338 kJ almonds/d (320 kcal/d) for 6 mo on body weight in men and women ranging in age from 25 to 70 yr and with a BMI <95th percentile. The almond supplement provided 15% of daily energy requirements for each person, and no dietary advice or recipes were provided. Subjects could incorporate the nuts as they wished. There was a nonsignificant weight gain of 0.4 kg for the group overall, but, when subjects were separated by sex, the men gained 0.65 kg (\( P < 0.01 \)), and the women gained only 0.11 kg (\( P = 0.79 \)). When the sexes were studied together, only persons in the lowest and middle tertiles of BMI gained weight. The estimated compensation for total EI was 78.2%. This study showed that incorporating nuts in a regular, free-living diet does not lead to weight gain; however, no concurrent group without the almond supplement was studied during that same period. It is therefore not known whether study participation had an influence on compliance or whether it helped improve dietary compensation for the added energy.

Another study examined the effects of peanut consumption on energy balance and the hedonic ratings for peanuts and other snack foods (70). Normal-weight men and women were given \( \approx 89 \text{ g peanuts/d}, which is equivalent to 2113 \text{ kJ/d (505 kcal/d)}, to consume as they wished for 8 wk (free-feeding phase); this phase was followed by a 3-wk phase during which the peanuts were added to the baseline diet (addition phase) and an 8-wk phase during which peanuts replaced fat in the diet (substitution phase). The phases of the study were separated by 4-wk washout periods. During the free-feeding phase, mean energy compensation was 66%, and the observed weight gain was 1.0 kg, which is significantly lower than the theoretical, expected weight gain of 3.6 kg. Weight gain (0.6 kg) was also significantly lower during the addition phase than predicted (1.4 kg), and there was no change in body weight during the substitution phase. Resting EE increased by 11% after 19 wk of regular peanut consumption, even after adjustment for changes in body weight. These results show that nut consumption may have a small effect on EE, which may partly explain the lower-than-expected weight gain observed in the free-feeding and addition phases.

Finally, results from nut supplementation studies do not imply that nut consumption can assist in weight loss, and there are no definitive data on their ability to assist in the maintenance of
stable weight. Nevertheless, their satiating power may play a role in weight maintenance. In addition, the Nurses’ Health Study (72) and the Seventh Day Adventist Study (73) found lower body weights with increased nut consumption. However, these cross-sectional studies may also reflect a healthier lifestyle pattern associated with nut consumption. Weight-loss studies incorporating nuts should be conducted to ascertain whether nuts may assist in weight loss. At present, available research allows us only to speculate that nuts may assist in controlling body weight, perhaps via increased satiety levels, increased resting EE, or energy malabsorption.

CONCLUSION

The studies reviewed here show that some foods have the potential to enhance weight loss or prevent weight gain. The body of literature seems to provide more support for the replacement of dietary LCTs with dietary MCTs or diacylglycerols than for the inclusion of other potential functional foods as part of the diet. However, available data suggest that beverages such as tea and milk may also be of value. Additional clinical studies are needed to ascertain whether tea and milk consumption can help improve body weight maintenance and perhaps also assist in weight loss. More research is also necessary to reach conclusions about the effects of nut consumption on body weight, and weight-loss studies with a control group are necessary to determine with greater accuracy the possible effects of all of the potential functional foods reviewed here. Such studies would, however, require a very large sample size because the effect size is expected to be small.

As yet, no weight-loss claim can be made for any of the foods examined here. Nevertheless, the data do suggest that incorporating some of these foods in a healthy and balanced diet could be beneficial for weight maintenance. Perhaps the individual effect of each dietary component on weight control might be too small to result in meaningful body-composition changes, but, if the dietary components were combined, their effects could be significant. It is nonetheless important that these measures be combined with energy restriction and increased physical activity to achieve significant weight loss.

The author has no personal or financial conflicts of interest with respect to the subject of this review.

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Beneficial metabolic effects of regular meal frequency on dietary thermogenesis, insulin sensitivity, and fasting lipid profiles in healthy obese women

Hamid R Farshchi, Moira A Taylor, and Ian A Macdonald

ABSTRACT

Background: Although a regular meal pattern is recommended for obese people, its effects on energy metabolism have not been examined.

Objective: We investigated whether a regular meal frequency affects energy intake (EI), energy expenditure, or circulating insulin, glucose, and lipid concentrations in healthy obese women.

Design: Ten women [3 ± SD body mass index (in kg/m²); 37.1 ± 4.8] participated in a randomized crossover trial. In phase 1 (14 d), the subjects consumed their normal diet on 6 occasions/d (regular meal pattern) or followed a variable meal frequency (3–9 meals/d, irregular meal pattern). In phase 2 (14 d), the subjects followed the alternative pattern. At the start and end of each phase, a test meal was fed, and blood glucose, lipid, and insulin concentrations were determined before and for 3 h after (glucose and insulin only) the test meal. Subjects recorded their food intake on 3 d during each phase. The thermogenic response to the test meal was ascertained by indirect calorimetry.

Results: Regular eating was associated with lower EI (P < 0.01), greater postprandial thermogenesis (P < 0.01), and lower fasting total (4.16 compared with 4.30 mmol/L; P < 0.01) and LDL (2.46 compared with 2.60 mmol/L; P < 0.02) cholesterol. Fasting glucose and insulin values were not affected by meal pattern, but peak insulin concentrations and area under the curve of insulin responses to the test meal were lower after the regular than after the irregular meal pattern (P < 0.01 and 0.02, respectively).

Conclusion: Regular eating has beneficial effects on fasting lipid and postprandial insulin profiles and thermogenesis.

KEY WORDS

Regular meal pattern, energy intake, energy expenditure, insulin, lipids

INTRODUCTION

The prevalence of obesity continues to rise, and cardiovascular disease (CVD) remains a major health problem throughout the world. Moreover, obesity can increase the risk of CVD. Meal frequency is one of a number of factors that may contribute to the development of obesity and CVD. In the 1960s, Fabry et al (1) suggested a negative association between meal frequency and body weight, and many investigators have subsequently attempted a further evaluation of this association (2–7). The results of studies evaluating the effect of meal frequency on energy intake (EI) were inconclusive (2, 4, 8–13). There appears to be no association between meal frequency and total energy expenditure (EE; 14–17), whereas the results of studies of the effect of meal frequency on the thermic effect of foods (TEF) were contradictory (18–21). However, no studies have considered the effects of day-to-day variations in meal frequency on the components of energy balance.

Altered circulating total, LDL-, and HDL-cholesterol; triacylglycerol (22–24); and insulin (25) concentrations are recognized as risk factors for CVD. Meal frequency may influence these factors, and an increased meal frequency is associated with lower fasting total and LDL-cholesterol concentrations (26–28). However, studies of the response of glucose and insulin to variations in meal frequency were inconclusive (1, 2). No studies have investigated the effect of irregular meal frequency on lipid metabolism.

It seems that Western populations increasingly are moving away from regular meals, perhaps because more meals are being eaten outside the home and because the tradition of families dining together has been eroded by hectic schedules. The prevalence of irregular meal patterns is greater among adolescents than it was during previous decades (29, 30). Japanese studies (31–33) also found that irregular snacking has become more common in children and may have contributed to both the increasing prevalence of obesity in children and the elevated serum cholesterol concentrations in adolescents during the past few decades.

We recently showed that an irregular meal frequency disturbs energy metabolism in healthy lean women (34). This irregular meal frequency led to a lower postprandial EE than was seen with regular eating, whereas mean EI did not differ significantly. We
also found a higher degree of insulin resistance and higher fasting lipid profiles in these lean subjects after a period of irregular meal frequency, which may indicate a deleterious effect on these CVD risk factors (35). It is not known whether an irregular meal pattern has similar effects in obese subjects. The purpose of the current study was to examine the effect of irregular meal frequency on EI, EE, and lipid and carbohydrate metabolism in healthy obese women.

SUBJECTS AND METHODS

Subjects

Ten healthy obese women aged 32–47 y (\( x \pm SD: 39.9 \pm 5.7 \) y) whose menstruation is regular or who are taking oral contraceptive pills, who are neither pregnant nor lactating, and who have no self-reported history of hypercholesterolemia, hyperglycemia, or any serious medical conditions were recruited from the general public by a notice in the local newspaper. Subjects were excluded if they reported that they were dieting [a score of \( >30 \)] on The Eating Inventory (36)] or experiencing depression [score of \( >10 \) on the Beck Depression Inventory (37)]. Mean body mass index (in kg/m\(^2\)) was 37.1 ± 4.8.

Ethical permission for the study was obtained from The University of Nottingham Medical School Research Ethics Committee. Written informed consent was obtained from all subjects.

Design

The randomized crossover trial consisted of 2 phases spanning a total of 42 d. Over the course of the study, the subjects made 4 laboratory visits and attended the screening session. In phase 1 (14 d), the subjects were asked to eat and drink items from their normal diet but either to consume the food and beverages on 6 occasions/d (regular meal pattern) with regular intervals between meals or to follow a chaotic meal plan (irregular meal pattern). To achieve the irregular meal pattern, the subjects were asked to observe a predetermined meal frequency consisting of 3–9 meals/d for 14 d, during which each number of meals per day was repeated twice (average: 6 meals/d). To achieve this, the subjects were asked to consume their usual diet on 7, 4, 9, 3, 5, 8, 6, 5, 9, 8, 3, 4, 7, and 6 occasions/d on days 1 through 14, respectively, of the irregular meal pattern. This irregular meal pattern is unlikely to precisely reflect free-living, chaotic eating, but standardization between subjects had to be ensured so that an effective comparison with the period of regular eating could be made. After phase 1, subjects were asked to consume their habitual diet and to follow their normal meal pattern for 14 d as a washout period. In phase 2 (14 d), the subjects were asked to cross over to the alternative meal pattern from phase 1. Five of the subjects started with the regular meal pattern, and 5 started with the irregular one. In both phases, a “meal” was defined as a food or snack (solid or liquid) containing energy, with an interval of \( >1 \) h between 2 eating occasions. The participants remained free-living during the study, and the diet content was self-selected.

Free-living food intake assessment

The participants were given training in keeping a food intake record by using a semiquantitative method based on household measurements. An instruction booklet with an example of a 1-d food intake record was given to each subject before each recording.

Before the start of phase 1, the subjects recorded their habitual dietary intake on 1 weekend day and 2 weekdays. The subjects were asked to provide a food intake record for 3 of the 14 d of the regular meal pattern phase to measure their adherence to this diet. They were also asked to record their dietary intake on the 3 corresponding days during the irregular meal pattern period, when they were eating 9, 6, and 3 meals/d. Food diaries of the subjects’ habitual, regular, and irregular meal patterns were analyzed by using MICRODIET software (version 1.2; Downlee Systems Limited, Salford, United Kingdom).

Protocol for laboratory visits

The subjects were asked to fast overnight (for \( \geq 10 \) h) and to take no exercise other than the walking required for the activities of daily living for 48 h before the laboratory visit. On the subjects’ arrival at the laboratory, their weight, height, and waist and hip circumferences were measured. Then, a 20-gauge cannula was inserted retrogradely into a dorsal hand vein for collection of arterialized venous blood. Two baseline blood samples were taken in the fasting state, a milkshake test meal was given, and blood samples were taken at 15-min intervals for 3 h. Resting metabolic rate (RMR) was measured in the fasting state and then for 3 h after the test meal. Subjects also completed visual analogue scales for hunger-related factors before and for 3 h after consuming the test meal. All visits were undertaken in the morning.

Laboratory procedures

Anthropometric measurements

Weight and waist and hip circumference measurements were made at each laboratory visit. Weight was measured to the nearest 0.1 kg on a Seca electronic scale (model no. 882; Vogel and Halke, Hamburg, Germany) when subjects were fasting, had an empty bladder, were wearing light clothing with empty pockets, and were not wearing shoes. Height was measured to the nearest 0.1 cm by using a stadiometer during the screening visit. Waist circumference was measured to the nearest 0.1 cm in a horizontal plane at the midpoint between the lower margin of the last rib and the crest of the ilium when the subject stood with her feet 25–30 cm apart (38). Hip circumference was also measured to the nearest 0.1 cm in a horizontal plane at the maximum point over the buttock at the level of the femoral greater trochanter by using a flexible nonstretch nylon tape (38). Body composition was measured by using bioelectrical impedance analysis (QuadScan 4000; Bodystat Ltd, Douglas, United Kingdom) while the subject lay on a nonconductive couch with the arms and legs abducted.

Blood sampling

On each subject’s arrival at the laboratory, her hand was warmed in a heated, ventilated perspex box (50–55 °C) for 15–20 min to open the arteriovenous anastomoses (39). A 20-gauge cannula was inserted retrogradely into a dorsal hand vein, and a slow-running infusion of saline (154 mmol sodium chloride/L) was begun to keep the cannula patent. Blood samples were withdrawn via a 3-way tap; the first 2 mL was discarded to
avoid contamination with saline. Two baseline blood samples were tested for fasting blood glucose, serum insulin, and plasma cholesterol (total, HDL, and LDL), triacylglycerol, and catecholamines. After the test meal, blood samples were taken every 15 min for 3 h. These blood samples were analyzed for blood glucose, serum insulin, and plasma catecholamines.

**Test meal consumption**

The milkshake test meal was given as a breakfast. Subjects were given a volume of test meal on the basis of their weight [12.8 kcal (53.5 kJ)/kg lean body mass]. Of the total energy from the macronutrients, 50% was as carbohydrate, 35% was as fat, and 15% was as protein. The test meal contained 2%–fat milk, a milkshake powder (Build-up; Nestlé SA, Lausanne, Switzerland), double cream (containing 1831 kJ energy and 47.5 g fat/100 mL, of which 29.7 g is saturated fat; Sainsbury’s, London, United Kingdom), and Polycal glucose polymer (Nutricia Clinical Care, Trowbridge, United Kingdom) in either strawberry or vanilla flavor. The milkshake test meal was served at a temperature of 18–20 °C in an open glass. Subjects were asked to consume the drink over 10 min.

**Visual analogue scales**

Each subject completed visual analogue scale questionnaires to assess subjective hunger, satiety, fullness, and prospective food consumption (ie, desire to eat). Subjects completed these questionnaires just before and every 30 min after the test meal for 3 h. Ratings were made on 100-mm visual analogue scales with words at each end that expressed the most extreme rating (40).

**Energy expenditure and substrate oxidation**

RMR was measured after a 10-h fast by using an open-circuit indirect calorimeter [Gas Exchange Measurement (GEM) system; Nutren Technology Ltd, Manchester, United Kingdom]. After a warm-up period of 30 min, a reference gas (5% CO2 and 95% O2) was used to calibrate the oxygen and carbon dioxide analyzers. Ingoing and outgoing air were analyzed for oxygen and carbon dioxide every minute during each period of measurement. Readings from the metabolic monitor were collected every minute with a personal computer.

The subjects rested on a bed in a room maintained at 18–20 °C for 20–20 min. Fasting RMR was then measured for 30 min with the subject lying in the supine position. A transparent ventilated hood was positioned over the subject’s head with Collins tubing connecting the hood to the monitor, and expired gases were continuously collected. The subjects then drank the milkshake test meal over a period of 10 min. Starting immediately after consumption of the test meal, postprandial metabolic rate (PPMR) was measured for 2 periods of 15 min every hour for 3 h. The subjects rested on the bed but were not allowed to sleep during the EE measurements. In the intervals between the measurements, subjects remained on the bed but were permitted to read. The TEF was measured by using the trapezoidal method to calculate the area under the curve (AUC) of PPMR above the baseline RMR for all of the visits.

**Processing of blood samples**

Immediately after blood samples were drawn, blood glucose was measured by using a B-Glucose analyzer (Hemocue AB, Angelholm, Sweden). After collection, blood samples for insulin analysis were left to clot for at least 30 min after collection before being centrifuged for 10 min at 3000 RPM (Minifie RF; Heraeus Equipment Ltd, Brentwood, United Kingdom). The serum samples were then sealed and stored at −80 °C for future analysis. Blood samples for cholesterol, triacylglycerol, and catecholamine analyses were taken into tubes containing lithium heparin. The samples were kept in an icebox until the end of each visit before being centrifuged for 10 min at 3000 RPM (Minifie RF; Heraeus Equipment Ltd). The plasma was transferred into a fresh tube containing 25 μL EGTA. The tube was then sealed and stored at −80 °C for later analysis of lipids, uric acid, and catecholamines.

Insulin measurements were performed by using a solid-phase [125I]-radioimmunoassay method and coated-tube technology (Count-A-Count; Diagnostic Products Corp, Los Angeles). The intraassay CV was 2.8% and 3.3% for blood glucose and serum insulin, respectively.

Plasma total cholesterol and triacylglycerol were measured enzymatically by using kits and standards supplied by VITROS (Ortho-Clinical Diagnostics, Rochester, NY). HDL-cholesterol concentrations were measured after precipitation of apolipoprotein B–containing lipoproteins with heparin and manganese chloride (41) by using the EZ HDL cholesterol kit (Sigma Diagnostics, St Louis). LDL-cholesterol concentrations were calculated by using the formula of Friedewald et al (42). Plasma uric acid concentrations were measured enzymatically (43, 44) by using kits supplied by VITROS. The intraassay CV was 2.2%, 2.6%, 1.7%, and 1.6% for total cholesterol, HDL cholesterol, triacylglycerol, and uric acid, respectively.

Homeostasis model assessment was used to assess insulin resistance (HOMA-IR) when the subjects were in the fasting state. The HOMA-IR values for the subjects were calculated by using the following formula (45):

\[
\text{HOMA-IR} = \frac{\text{fasting serum insulin (μIU/mL)}}{\text{fasting blood glucose (mmol/L)/22.5}}
\]

HPLC with electrochemical detection was used to measure plasma epinephrine and norepinephrine concentrations (46). AUCs above the baseline for blood glucose, serum insulin, and plasma norepinephrine concentrations were measured during the 3-h posttest meal period by using the trapezoidal method.

**Statistical analysis**

SPSS software (version 10; SPSS, Chicago) was used for data entry and analysis. All data are reported as means ± SDs unless otherwise indicated. Data were tested for normality (Kolmogorov-Smirnov statistic with Lilliefors correction). Comparisons of the preintervention data were performed by using Student’s paired t test (two-tailed) to investigate possible differences before the meal pattern interventions. Statistical analysis of the results was then performed by using repeated-measures analysis of variance (ANOVA) with 2 within-subjects factors (ie, visits before and after the interventions and the meal pattern) or with 3 within-subjects factors (ie, meal pattern, values before and after the interventions, and the time after the test meal) as appropriate. When ANOVA indicated an interaction (P < 0.10) between the factors, paired t tests were undertaken between the postintervention results and between the preintervention and
postintervention values. In the absence of an interaction, significant main effects (detected by ANOVA) are reported. Pearson correlation analysis was performed between differences in energy intake and the observed metabolic changes. Significance was set at $P < 0.05$ for all statistical tests.

RESULTS

There were no differences between the preintervention and postintervention body weights under either meal pattern (ANOVA). There were also no significant differences between preintervention and postintervention anthropometric measurements or body-fat composition under either meal pattern period (ANOVA; Table 1).

Energy intake

The preexperiment food diaries did not show any significant difference in EI between the average of the 2 weekdays (8.43 ± 1.04 MJ/d) and the 1 weekend day (8.54 ± 1.02 MJ/d). Furthermore, there were no significant differences in the macronutrient composition of the food between weekdays and weekends.

All subjects reported having adhered to the appropriate meal patterns during the 2 interventions. Mean energy intake recorded over 3 d was significantly lower during the regular meal pattern (7.98 ± 0.49 MJ/d) than during the irregular meal pattern (8.32 ± 0.35 MJ/d; $P < 0.01$). EI did not differ significantly between the 3 d of the regular meal pattern, but EI values did differ significantly between all of the days with 9, 6, and 3 meals/d during the irregular meal pattern (ANOVA; $P = 0.017$). EI was significantly higher with 9 meals/d than with 3 meals/d (paired $t$ test; $P = 0.012$), but there was no significant difference between 9 and 6 meals/d or between 6 and 3 meals/d (Table 2). There were no significant differences in macronutrient composition (ie, percentage of energy from protein, fat, and carbohydrate) between the 2 meal patterns or between the days within each meal pattern period (Table 2).

APPETITE MEASUREMENT

The response curves for the 4 appetite sensations (ie, hunger, satiety, fullness, and prospective food consumption) are shown in Figure 1. Fasting values for all variables and the profiles after the test meal did not differ significantly over the course of the experiment.

Energy expenditure and thermic effect of food

There was no significant difference in the fasting RMR values between the visits before the regular and the irregular meal pattern periods (paired $t$ test), and there was no significant effect of either meal pattern on fasting RMR (by ANOVA; Figure 2). Metabolic rate increased significantly above fasting values after the test meal at all visits. The PPMR response showed a significant 3-way meal pattern–by–visit (before and after intervention)–by–time after the test meal interaction (ANOVA; $P = 0.03$). PPMR showed no significant difference between the preintervention visits; however, PPMR was significantly lower after the irregular meal pattern than before it and significantly higher after the regular meal pattern than before it (Figure 2). There was a significant meal pattern–by–visit (before and after intervention) interaction for the TEF response (ANOVA; $P = 0.002$; Figure 3). The TEF at the visits before the 2 periods did not differ significantly, but the change in TEF differed significantly between the intervention periods: TEF fell significantly after the irregular meal pattern (paired $t$ test; $P = 0.008$), but it rose significantly after the regular meal pattern (paired $t$ test; $P = 0.018$).

TABLE 1
Physical characteristics of the 10 subjects during the experiment

<table>
<thead>
<tr>
<th></th>
<th>Regular meal pattern Before intervention</th>
<th>After intervention</th>
<th>Irregular meal pattern Before intervention</th>
<th>After intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>98.5 ± 15.6</td>
<td>98.1 ± 15.4</td>
<td>98.1 ± 15.3</td>
<td>98.4 ± 15.8</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>37.1 ± 4.8</td>
<td>36.9 ± 4.8</td>
<td>36.9 ± 4.9</td>
<td>37.0 ± 4.9</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>102.8 ± 12.1</td>
<td>103.8 ± 12.9</td>
<td>103.1 ± 10.6</td>
<td>104.1 ± 12.1</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.85 ± 0.04</td>
<td>0.84 ± 0.03</td>
<td>0.85 ± 0.05</td>
<td>0.84 ± 0.04</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>45.0 ± 3.3</td>
<td>44.7 ± 3.0</td>
<td>45.1 ± 3.0</td>
<td>45.3 ± 2.9</td>
</tr>
</tbody>
</table>

1 All values are $\bar{x} \pm SD$. There were no significant differences in physical characteristics during the experiment (ANOVA).

TABLE 2
Daily nutrient intakes of the 10 subjects during the regular and irregular meal patterns

<table>
<thead>
<tr>
<th></th>
<th>Regular meal pattern Day 1 (6 meals)</th>
<th>Day 2 (6 meals)</th>
<th>Day 3 (6 meals)</th>
<th>Overall</th>
<th>Irregular meal pattern Day 1 (9 meals)</th>
<th>Day 2 (3 meals)</th>
<th>Day 3 (6 meals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (MJ)</td>
<td>7.97 ± 0.61</td>
<td>7.95 ± 0.63</td>
<td>8.01 ± 0.52</td>
<td>7.98 ± 0.49</td>
<td>8.82 ± 0.65</td>
<td>7.77 ± 0.56</td>
<td>8.33 ± 0.84</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>17.6 ± 4.9</td>
<td>16.2 ± 3.5</td>
<td>15.8 ± 5.1</td>
<td>16.5 ± 2.5</td>
<td>15.7 ± 2.1</td>
<td>16.9 ± 2.9</td>
<td>14.8 ± 3.4</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>38.5 ± 4.1</td>
<td>38.3 ± 6.3</td>
<td>36.9 ± 6.4</td>
<td>37.9 ± 3.2</td>
<td>35.7 ± 5.9</td>
<td>38.7 ± 5.9</td>
<td>40.9 ± 6.5</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>43.7 ± 4.5</td>
<td>43.9 ± 6.5</td>
<td>46.3 ± 8.1</td>
<td>44.6 ± 2.8</td>
<td>46.0 ± 4.7</td>
<td>42.0 ± 6.1</td>
<td>43.7 ± 8.2</td>
</tr>
</tbody>
</table>

1 $P = 0.07$ for interaction of pattern and days based on ANOVA.

2 Significantly different between 9, 3, and 6 meals/d, $P = 0.017$ (ANOVA); significantly higher with 9 meals/d than with 3 meals/d, $P = 0.012$ (paired $t$ test); no significant difference between 6 and 9 meals/d or between 6 and 3 meals/d.

3 Significantly different from regular meal pattern, $P = 0.005$ (paired $t$ test).
Blood glucose and serum insulin

There was no significant difference in fasting blood glucose between the visits before the regular and irregular meal pattern periods (paired t test). There was no significant effect of either meal pattern on fasting blood glucose (ANOVA; Table 3). Blood glucose concentrations rose significantly after the test meal at all visits (Figure 4). The preintervention peak in blood glucose concentrations did not differ between meal pattern interventions.
TABLE 3
Fasting glucose, insulin, lipid, uric acid, and norepinephrine concentrations and peak postprandial glucose and insulin concentrations in the 10 subjects during the experiment

<table>
<thead>
<tr>
<th></th>
<th>Regular meal pattern</th>
<th>Irregular meal pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before intervention</td>
<td>After intervention</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/L)</td>
<td>5.06 ± 0.15</td>
<td>5.02 ± 0.17</td>
</tr>
<tr>
<td>Fasting serum insulin (pmol/L)</td>
<td>130 ± 54</td>
<td>122 ± 42</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>4.91 ± 2.16</td>
<td>4.55 ± 1.60</td>
</tr>
<tr>
<td>Plasma cholesterol (mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.28 ± 0.50</td>
<td>4.16 ± 0.46</td>
</tr>
<tr>
<td>HDL</td>
<td>1.45 ± 0.25</td>
<td>1.46 ± 0.26</td>
</tr>
<tr>
<td>LDL</td>
<td>2.59 ± 0.64</td>
<td>2.43 ± 0.63</td>
</tr>
<tr>
<td>Plasma triacylglycerol (mmol/L)</td>
<td>1.21 ± 0.34</td>
<td>1.16 ± 0.51</td>
</tr>
<tr>
<td>Plasma uric acid (mg/L)</td>
<td>239 ± 29</td>
<td>237 ± 31</td>
</tr>
<tr>
<td>Plasma norepinephrine (nmol/L)</td>
<td>1.18 ± 0.43</td>
<td>1.20 ± 0.38</td>
</tr>
<tr>
<td>Peak of postprandial blood glucose (mmol/L)</td>
<td>7.50 ± 0.41</td>
<td>7.44 ± 0.40</td>
</tr>
<tr>
<td>Peak of postprandial serum insulin (pmol/L)</td>
<td>843 ± 274</td>
<td>807 ± 275</td>
</tr>
</tbody>
</table>

1 All values are ¯ ± SD. HOMA-IR, homeostasis model assessment of insulin resistance.
2 Significantly different from the regular meal pattern, P = 0.003 (paired t test).
3 A significant meal pattern–by–visit (before and after intervention) interaction was observed for plasma total cholesterol concentrations, P = 0.002 (ANOVA).
4 Significantly different from the regular meal pattern intervention, P = 0.038 (paired t test).
5 A significant meal pattern–by–visit (before and after intervention) interaction was observed for plasma LDL concentrations, P = 0.013 (ANOVA).
6 Significantly different from the irregular meal pattern intervention, P = 0.005 (ANOVA).

FIGURE 4. Mean (±SEM) blood glucose concentrations in 10 healthy obese women in the fasting state and after the test meal fed at the visits before and after the regular and irregular meal pattern interventions. No significant differences were observed in blood glucose profiles over the course of the experiment. For clarity, SEM values are presented for only 2 of the profiles.

MEAN VALUES OF FASTING PLASMA TOTAL, LDL, AND HDL CHOLESTEROL AND TRIACYLGLYCE Rol concentrations in the 10 subjects during the experiment (ANOVA; P = 0.005). Peak of serum insulin fell significantly after the regular meal pattern intervention (paired t test; P = 0.001), but it rose significantly after the irregular meal pattern intervention, P = 0.021 (paired t test).

There was no significant difference in HOMA-IR values between the visits before the regular and irregular meal pattern interventions, and HOMA-IR did not change significantly after either meal pattern intervention (Table 3).

Serum insulin concentrations rose significantly in response to the test meal at all visits. There was no significant difference in the peak insulin values after the test meal between the visits before the regular and irregular meal pattern interventions. There was a significant meal pattern–by–visit (before and after intervention) interaction for the peak serum insulin concentrations (ANOVA; P = 0.005). Peak of serum insulin fell significantly after the regular meal pattern intervention (paired t test; P = 0.001) compared with a significant rise after the irregular meal pattern intervention (paired t test; P = 0.021; Table 3). There was no significant difference in the AUC of postprandial insulin between the visits before the regular and irregular meal pattern interventions, but there was a significant meal pattern–by–visit (before and after intervention) interaction (ANOVA; P = 0.017) for the AUC of insulin response (Figure 5). The AUC of serum insulin response fell significantly after the regular meal pattern intervention (paired t test; P = 0.022), whereas it rose significantly after the irregular meal pattern intervention (paired t test; P = 0.022).

Plasma lipids and uric acid

The mean values of fasting plasma total, LDL, and HDL cholesterol and triacylglycerol concentrations at all visits are also shown in Table 3. The lipid values did not differ significantly between the visits before the 2 meal pattern interventions. There was a significant meal pattern–by–visit (before and after intervention) interaction for fasting HDL concentrations (ANOVA; P = 0.002). Plasma total cholesterol was also significantly lower after the regular meal pattern intervention than after the irregular meal pattern intervention (paired t test; P = 0.003). There was also a significant meal pattern–by–visit (before and
after intervention) interaction for plasma LDL-cholesterol concentrations (ANOVA; \( P = 0.013 \)). In addition, the plasma LDL concentrations were significantly lower than after the regular meal pattern intervention than after the irregular meal pattern intervention (paired \( t \) test; \( P = 0.038 \)). However, no significant differences were observed in plasma HDL concentrations during either meal pattern intervention. Plasma triacylglycerol concentrations also showed no significant difference over the course of the experiment.

There were no significant correlations between the changes in total or LDL cholesterol and the differences in mean energy intake between the regular and irregular meal pattern interventions. There were also no significant correlations between the differences in EI and the insulin and TEF responses. There were no significant changes in plasma uric acid over the course of the experiment (Table 3).

**Plasma norepinephrine**

There was no significant difference in fasting plasma norepinephrine concentrations between the 2 preintervention visits (paired \( t \) test). There was no effect of either meal pattern on fasting plasma norepinephrine (ANOVA; Table 3). Plasma norepinephrine concentration rose significantly after the test meal at the visits before and after the regular meal pattern intervention. No significant differences were observed in plasma norepinephrine profiles over the course of the experiment. For clarity, SEM values are presented for only 2 of the profiles.

The peak plasma norepinephrine concentrations before each intervention were not significantly different (paired \( t \) test) and were not significantly affected by meal pattern (ANOVA). There was no significant difference in the AUC of norepinephrine profiles above the baseline between the visits before the 2 meal pattern interventions. There was also no effect of either meal pattern intervention on the AUC of plasma norepinephrine profiles over the course of the study (ANOVA; Figure 6).

Plasma epinephrine concentrations were 0.05–0.21 nmol/L at baseline, and there was no significant difference between the 2 preintervention visits. Epinephrine concentrations did not change significantly during the postprandial periods (data not shown).

**DISCUSSION**

The aim of this study was to investigate the effect of irregular meal frequency on EI, resting EE, and indexes of carbohydrate and lipid metabolism and plasma uric acid concentration. We found that irregular meal frequency led to a lower postprandial EE and impaired TEF, higher total and LDL cholesterol, and lower postprandial insulin sensitivity than were seen with regular meal frequency in healthy obese women; these findings were in agreement with those of our earlier study in lean women (34, 35). We did not find any significant difference in fasting RMR across the 4 visits. As explained earlier, many studies since the 1960s have evaluated the effect of meal frequency on EI and EE. The poor definition of key variables and the lack of the management of the intervention factors have weakened their interpretations. In the current study, we clearly defined a meal as providing some energy, and the interval between any 2 consecutive meals was to be \( \geq 1 \) h. Fluctuations have been reported in women’s food intake (47) and RMR (48) through the menstrual cycle. To overcome this possible factor, each subject began the interventions at the same point in her menstrual cycle.

Previous studies showed that impaired thermogenesis is associated with insulin resistance in obesity (49, 50), which is consistent with the current finding of lower TEF and postprandial insulin insensitivity after the irregular meal pattern intervention. Another study (51) found an independent effect of insulin resistance and obesity in producing a blunted TEF. In the current study, the irregular meal pattern intervention failed to produce any significant differences in body weight, despite reduced dietary thermogenesis, but this was to be expected because of the short duration of the intervention. However, insulin insensitivity and the low TEF resulting from the irregular meal pattern intervention are in agreement with the findings of previous studies of
an association between insulin resistance and blunted thermogenesis. There is some evidence of a link between sympathetic nervous system activation and TEF response to a meal in younger subjects but not in older subjects (52). Despite a significantly lower TEF after the irregular meal pattern intervention than after the regular meal pattern intervention, we did not find any significant differences in plasma norepinephrine concentration (an index of sympathetic nervous system activity) over the course of the experiment, although there was a trend for a higher response after the regular meal pattern intervention. Further investigation is required to ascertain the mechanism of a lower TEF in response to an irregular meal pattern intervention.

Several studies (53–55) found that underreporting of EI, especially for snacks, was more common in obese persons. The current study may also have been affected by underreporting, but we assume that the degree of underreporting was similar in all phases. Despite instructions to maintain a normal food intake, the subjects reported a significantly higher mean EI during the irregular meal pattern intervention than during the regular meal pattern intervention. In our earlier study with the same experimental design, lean women showed no significant differences in EI between regular and irregular meal pattern interventions (34). That indicates that obese persons consuming self-selected diets may have found it more difficult to adjust to an irregular meal frequency and also maintain their normal intake of foods. There may also be some inaccuracy in extrapolating the EI record for 3 d to represent the entire 14-d intervention period. The EI of the subjects in the irregular meal pattern intervention differed significantly between the days with 9, 6, and 3 meals/d. EI was significantly higher with 9 meals/d than with 3 meals/d. This is in agreement with the findings of one earlier study (8), but not with those of other studies (9–12). An intervention study (13) also reported no association between meal frequency and EI. The current study showed no significant differences between the 2 meal pattern interventions in the appetite ratings after the test meal. Further investigation in a feeding study combined with appetite ratings would be essential to ascertain the effect of an irregular meal pattern on total EI and appetite control.

The effect of a meal pattern, and especially of meal frequency, on the biochemical risk factors for CVD has been of interest to many researchers (26–28), but the results are not conclusive. A study (56) also indicated an inverse relation between meal frequency and serum uric acid concentration. The HOMA-IR index showed no significant differences in the current study, which indicated no overall change in fasting insulin sensitivity in these obese subjects. This finding is somewhat different from the results of the HOMA-IR index seen previously in nonobese subjects (35). It is possible that, because the obese subjects are likely to already have some degree of insulin resistance, this metabolic factor may not be exacerbated in obese subjects during the 2-wk intervention as much as it would be in nonobese subjects with greater initial fasting insulin sensitivity. The peak postprandial insulin response and AUC of the insulin profile after the test meal in the obese subjects were significantly lower after the regular meal pattern intervention than after the irregular meal pattern intervention, which is consistent with findings of our earlier study of lean women (35). This finding leads us to propose that an irregular meal frequency may reduce insulin sensitivity and thus lead to a higher insulin response to a test meal, whereas a regular meal frequency may increase insulin sensitivity. The molecular basis of these changes remains to be determined. The effect of a particular meal pattern on postprandial insulin sensitivity may carry over to the fasting state. In the current study, plasma total and LDL-cholesterol concentrations were significantly higher after the irregular meal pattern intervention than after the regular meal pattern intervention. The differences in total and LDL-cholesterol concentrations (and in insulin sensitivity and TEF) were not correlated with the differences in EI. This lack of correlation may be due to a lack of statistical power, but it suggests that meal pattern is likely to have a greater metabolic effect than small differences in EI. Fasting plasma HDL-cholesterol, triacylglycerol, and uric acid concentrations did not differ significantly between the regular and irregular meal pattern interventions. These results are in agreement with those of our earlier study of nonobese women (35). These 2 studies indicate that irregular eating may lead to unhealthy total and LDL-cholesterol concentrations in both obese and nonobese women. Previous studies showed that a greater meal frequency is associated with lower fasting total and LDL-cholesterol concentrations. An epidemiologic study (33) claimed that an irregular meal pattern may be associated with an elevated serum total cholesterol concentration in adolescents. However, the report of that study did not provide an exact definition of an irregular meal pattern.

The current study showed the importance of meal pattern in addition to the amount and composition of food in influencing carbohydrate and lipid metabolism. Further studies measuring the above factors and other possible influences are required. The current study also showed lower TEF and higher EI with an irregular meal frequency. This indicates a potential mechanism by which an irregular meal pattern might affect EE and EI, which could lead to weight gain in the longer term. In addition, the irregular meal pattern had potentially deleterious effects on insulin sensitivity and plasma cholesterol, which are known risk factors of CVD.

We thank the women who participated in the study. We gratefully acknowledge the technical assistance of Liz Simpson and Heather Sissons throughout the study. We thank the Clinical Chemistry Laboratory (Queen’s Medical Centre, Nottingham, United Kingdom) for performing the lipid and uric acid assays, and we are grateful to Sally Cordon and Sarir Sarmad for performing the catecholamine assays.

HRF produced the initial study design, performed the laboratory investigations and biochemical analysis, undertook the statistical analysis, and wrote the first draft of the manuscript. MAT and IAM refined the study design and supervised the data collection, contributed to the data interpretation and re-drafting of the manuscript. MAT provided clinical and dietetic oversight to the dietary aspects of the study. None of the authors had any conflicts of interest.

REFERENCES

Greater enrichment of triacylglycerol-rich lipoproteins with apolipoproteins E and C-III after meals rich in saturated fatty acids than after meals rich in unsaturated fatty acids\(^1\text{–}^3\)

**Kim G Jackson, Emma J Wolstencroft, Paul A Bateman, Parveen Yaqoob, and Christine M Williams**

**ABSTRACT**

**Background:** Although there is considerable interest in the postprandial events involved in the absorption of dietary fats and the subsequent metabolism of diet-derived triacylglycerol-rich lipoproteins, little is known about the effects of meal fatty acids on the composition of these particles.

**Objective:** We examined the effect of meal fatty acids on the lipid and apolipoprotein contents of triacylglycerol-rich lipoproteins.

**Design:** Ten normolipidemic men received in random order a mixed meal containing 50 g of a mixture of palm oil and cocoa butter [rich in saturated fatty acids (SFAs)], safflower oil [n-6 polyunsaturated fatty acids (PUFAs)], or olive oil [monounsaturated fatty acids (MUFA)] on 3 occasions. Fasting and postprandial apolipoproteins B-48, B-100, E, C-II, and C-III and lipids (triacylglycerol and cholesterol) were measured in plasma fractions with Svedberg flotation rates (Sf) 400, Sf 60–400, and Sf 20–60.

**Results:** Calculation of the composition of the triacylglycerol-rich lipoproteins (expressed per mole of apolipoprotein B) showed notable differences in the lipid and apolipoprotein contents of the SFA-enriched particles in the Sf > 400 and Sf 60–400 fractions. After the SFA meal, triacylglycerol-rich lipoproteins in these fractions showed significantly greater amounts of triacylglycerol and of apolipoproteins C-II (Sf 60–400 fraction only), C-III, and E than were found after the MUFA meal (P < 0.02) and more cholesterol, apolipoprotein C-III (Sf > 400 fraction only), and apolipoprotein E than after the PUFA meal (P < 0.02).

**Conclusions:** Differences in the composition of Sf > 400 and Sf 60–400 triacylglycerol-rich lipoproteins formed after saturated compared with unsaturated fatty acid–rich meals may explain differences in the metabolic handling of dietary fats.

**KEY WORDS** Apolipoprotein B-100, apolipoprotein B-48, apolipoprotein C-II, n-6 polyunsaturated fatty acids, monounsaturated fatty acids

**INTRODUCTION**

Increased postprandial concentrations of triacylglycerol-rich lipoproteins, which circulate after a meal, are positively correlated with coronary heart disease risk (1, 2). As a result, there is considerable interest in the events involved in the absorption of dietary fats and the subsequent postprandial metabolism of the diet-derived triacylglycerol-rich lipoproteins (3). The type of fat consumed in a meal has been shown to influence the fatty acid composition of chylomicron particles and the subsequent postprandial triacylglycerol response (4). This finding suggests that meal fatty acid composition may influence the absorption, synthesis, secretion, and subsequent metabolism of dietary triacylglycerol. Unsaturated fatty acids have been shown to increase the size of chylomicron particles compared with saturated fatty acids (SFAs) (5, 6), with animal studies observing a more rapid hydrolysis of polyunsaturated fatty acid (PUFA)-enriched particles by lipoprotein lipase (LPL; EC 3.1.1.34) (7) and clearance of remnant particles by the liver (8). However, in those studies, differences in the rates of clearance of PUFA-enriched compared with monounsaturated fatty acid (MUFA)-enriched and SFA-enriched particles from the circulation could not be explained by differences in the activities of LPL and hepatic lipase (HL; EC 3.1.1.3) alone.

The metabolic handling of triacylglycerol-rich lipoproteins is known to be influenced by their apolipoprotein (apo) C and apo E composition, because these proteins have several regulatory functions. Apo C-II, which is synthesized in the liver and intestine, activates LPL, which hydrolyzes triacylglycerol within the core of the lipoprotein particles and plays a key role in the regulation of triacylglycerol clearance (9). Apo C-III, which is also synthesized in the liver and intestine, is thought to play several roles in the metabolism of triacylglycerol-rich lipoproteins. Increased concentrations of apo C-III have been shown to inhibit the binding and hydrolysis of lipoproteins by LPL and HL (9, 10) and to inhibit the recognition and uptake of lipoproteins by the liver (11, 12). Apo E, which is synthesized in the liver, plays a crucial role in mediating the hepatic recognition and uptake of the remnant particles by receptor-mediated processes (13). In addition, the overexpression of apo E on lipoproteins has been shown to inhibit the LPL hydrolysis of triacylglycerol-rich emulsions in vivo and in vitro (14). It has been suggested that, during their lifetime in the circulation, triacylglycerol-rich lipoproteins will

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contain multiple copies of these exchangeable apolipoproteins (15–17), the content of which will determine their metabolic fate in the circulation.

Although the apo C and E composition of different VLDL subfractions has been determined after a single oral fat load (18, 19), little is known about the compositional characteristics of triacylglycerol-rich lipoproteins after meals enriched with different fatty acids. Of the few studies conducted to date, apolipoprotein concentrations have been determined after emulsions enriched in PUFA and MUFA (apo C-II) (20) and after meals enriched in SFA and PUFA (21–23).

The aim of the present study was to quantify lipid and apolipoprotein (B, C-II, C-III, and E) concentrations in lipid fractions with a Svedberg flotation rate (Sf) > 400, Sf 60–400, and Sf 20–60 after meals enriched in SFAs, n–6 PUFAs, and n–9 MUFAs. We wished to determine whether differences in apolipoprotein content of triacylglycerol-rich lipoproteins could underlie differences in their metabolic handling after meals of various fatty acid compositions.

**SUBJECTS AND METHODS**

**Subjects**

Ten healthy middle-aged men [age (\(\bar{x}\) ± SD): 48 ± 9 y; body mass index (in kg/m^2\)) 25 ± 3] were studied on 3 occasions. Ethical consent was provided by The University of Reading Ethics Committee, and written informed consent was obtained from the subjects before the study began. Subjects were excluded if they had any metabolic disorders (eg, diabetes or any other endocrine or liver diseases), were taking dietary supplements (eg, fish oil), were smokers, were regular exercisers (>3 × 30 min of aerobic exercise per week), or were taking medication that could affect lipoprotein metabolism. The subjects were recruited after screening for fasting plasma triacylglycerol, cholesterol, and glucose concentrations, all of which were within normal limits (triacylglycerol: 1.3 ± 0.4 mmol/L; cholesterol: 5.0 ± 0.7 mmol/L; glucose: 5.7 ± 0.4 mmol/L). The subjects were asked to maintain their usual exercise patterns and to abstain from alcohol and organized exercise regimens for 24 h before each postprandial investigation. A low-fat evening meal was consumed on the evening before each study day.

The design of the study was a single-blind, within-subject crossover in which the subjects attended an investigation unit at The University of Reading on 3 separate occasions separated by at least 1 mo. Three test meals of different fatty acid composition were given to the subjects at 0800 in the form of a warm chocolate milkshake, breakfast cereal (Tesco, Cheshunt, UK), or white bread (105 g) and jam (30 g). The test meals were consumed ad libitum. The test meals were well tolerated by the subjects without any unpleasant side effects.

**Plasma separation and analytic methods**

Blood samples were transferred to heparin-containing tubes for the analysis of cholesterol, triglycerides, apo B-48, apo B-100, apo C-II, apo C-III, and apo E in the Sf > 400, Sf 60–400, and Sf 20–60 triacylglycerol-rich lipoprotein fractions. Plasma was separated by centrifugation at 1700 × g for 15 min in a bench-top centrifuge at 4 °C. EDTA (0.5 mmol/L), phenylmethylsulfonyl fluoride (10 mmol/L, dissolved in propan-2-ol), and aprotinin (10 000 kallikrein inactivator units/mL; Trasylol; Bayer plc, Newbury, United Kingdom) were added immediately to the isolated plasma to prevent the proteolytic degradation of the apolipoproteins. The plasma was stored overnight at 4 °C until isolation of the triacylglycerol-rich lipoprotein fractions by using density gradient ultracentrifugation (25) as previously described (26). After the triacylglycerol-rich lipoprotein fractions were collected (≈1 mL), they were divided into portions and stored at −20 °C until analyzed. To further protect the apolipoproteins from proteolytic cleavage, another preserving cocktail was added to the appropriate tubes before addition of the lipoprotein fractions to give a final concentration of 5% by vol (27).

Triacylglycerol and cholesterol concentrations were measured with an ILAB 600 clinical chemistry analyzer (Instrumentation Laboratory, Warrington, United Kingdom) by using

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Fat</th>
<th>Protein</th>
<th>Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>g</td>
<td>g</td>
<td>g</td>
<td>kJ</td>
</tr>
<tr>
<td>Test oil (50 g)</td>
<td>0</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Skimmed milk (150 g)</td>
<td>7.5</td>
<td>0.2</td>
<td>5.1</td>
</tr>
<tr>
<td>Nesquik (15 g)</td>
<td>12</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Skimmed milk powder (15 g)</td>
<td>7.8</td>
<td>0.2</td>
<td>5.3</td>
</tr>
<tr>
<td>White bread (105 g)</td>
<td>73.8</td>
<td>2.3</td>
<td>12.2</td>
</tr>
<tr>
<td>Jam (30 g)</td>
<td>27.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>128.6</td>
<td>55.2</td>
<td>23.1</td>
</tr>
</tbody>
</table>

^1 Determined from manufacturers’ data and from food tables (24). Nesquik is a chocolate-flavored milkshake mix manufactured by Nestlé (Vevey, Switzerland).

**TABLE 2**

Fatty acid composition of the test meals

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Fat</th>
<th>Protein</th>
<th>Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA meal</td>
<td>PUFA meal</td>
<td>MUFA meal</td>
<td></td>
</tr>
<tr>
<td>g/50 g</td>
<td>g</td>
<td>g</td>
<td>g</td>
</tr>
<tr>
<td>SFAs</td>
<td>26.1</td>
<td>7.8</td>
<td>7.1</td>
</tr>
<tr>
<td>PUFAs</td>
<td>3.2</td>
<td>31.8</td>
<td>5.9</td>
</tr>
<tr>
<td>MUFAs</td>
<td>19.5</td>
<td>8.2</td>
<td>34.9</td>
</tr>
</tbody>
</table>

^1 Determined from a computerized food database (FOODBASE; The Institute of Brain Chemistry and Human Nutrition, London) and manufacturers’ data. SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid.

**TABLE 1**

Nutritional composition of the test meals

Determined from manufacturers’ data and from food tables (24). Nesquik is a chocolate-flavored milkshake mix manufactured by Nestlé (Vevey, Switzerland).
enzyme-based colorimetric kits supplied by Instrumentation Laboratory. Apo C-II, C-III, and E were measured by the use of turbidimetric immunoassay using kits supplied by Alpha Laboratories (Eastleigh, Hants, United Kingdom). All samples for each subject were analyzed within a single batch, and the interassay CVs were <6%.

Apo B-48 was measured by a specific competitive enzyme-linked immunosorbent assay (ELISA; 28) as previously described (26). [Results with the apo B-48 heptapeptide standard yield apo B-48 concentrations that are ≈2.5 fold lower than those obtained by using human lymph as the standard.] Apo B-100 was measured by using a specific in-house sandwich ELISA. The inner 60 wells of a Nunc Maxisorp microtitre plate (Nunc, Roskilde, Denmark) were coated with 1 mg/mL of a 1:1 mixture of the apo B-100 monoclonal antibodies 4G3 and 5E11 (Ottawa Heart Institute Research Corporation, University of Ottawa Heart Institute, Ottawa) in 0.1 mol bicarbonate-carbonate buffer/L (pH 9.6) for 16 h at 4 °C. After washing with 0.02 mol phosphate-buffered saline (PBS)/L containing 0.05% (by vol) Tween 20 and 0.01% bovine serum albumin (PBSBT), the plate was blocked with 150 μL of 0.02 mol PBS/L, 0.025% (by vol) Tween 20, and 3% bovine serum albumin at 37 °C for 1 h. The wells in the microtitre plate were then emptied to receive the sample, and subsequent incubations were at 37 °C unless otherwise stated; all washing steps used PBSBT.

A 9-point standard curve was prepared by serial dilution of human LDL (density = 1.019–1.063 g/mL; Autobio Bioclear UK Limited, Wiltshire, United Kingdom) in PBSBT, thus producing a concentration range of apo B-100 from 1.25 mg/mL to 5 mg/mL. Pooled fasting plasma samples were collected to prepare the quality controls. The triacylglycerol-rich samples from the Sf 20–60 lipoprotein fraction were diluted 1:6, Sf 60–400 samples were diluted 1:1000, and Sf 20–60 samples were diluted 1:2000 in PBSBT before addition to the plate. Standards, samples, and quality controls (100 μL) were added in duplicate to the appropriate wells on the microtitre plate and were incubated for 90 min. The plates were washed, 100 μL of goat anti–apo B antibody (Guildhay Ltd, Surrey, United Kingdom) was added to the plate at a final dilution of 1:50 000, and the plate was incubated for a further 90 min. The plates were washed before the addition of the third antibody (anti–sheep-goat antibody conjugated to horseradish peroxidase; The Binding Site, Birmingham, United Kingdom) at a final dilution of 1:10 000. The plate was incubated for a further 90 min, was washed, and 100 μL of 3.3,5,5′-tetramethylbenzidine substrate (Sigma, St Louis) was added for 30 min. After the reaction had been stopped by the addition of 50 μL of 1 mol HCl/L, absorbance was read on an automated ELISA plate spectrophotometer (Tecan, Theale, United Kingdom) at 450 nm. A standard curve was constructed, and the amount of apo B-100 within the quality controls and the samples was determined. The interassay CVs for both the apo B-48 and the apo B-100 analyses were <10%.

Calculations

The number of apolipoprotein and lipid molecules per apo B particle was calculated by dividing apolipoprotein and lipid concentrations in each lipoprotein fraction by their respective molecular mass (apo B-100 = 549 kD, apo B-48 heptapeptide = 0.98 kD,apo C-II = 8.9 kD, apo C-III = 8.9 kD, and apo E = 36.5 kD). Because each of the triacylglycerol-rich lipoprotein fractions contained a mixture of apo B-48– and apo B-100–containing lipoproteins, the molarity of the apolipoproteins and lipids was then divided by the molarity of total apo B in each lipoprotein fraction (apo B-48 and apo B-100 concentrations combined).

Statistical analysis

The data were analyzed by using SPSS version 11 (SPSS Inc, Chicago). The results presented in postprandial time courses and in the tables are mean values ± SEMs. The area and incremental area under the curve (AUC and IAUC, respectively) were calculated by using the trapezoidal rule (29). Fasting concentrations, AUCs, and IAUCs were analyzed by using one-way repeated-measures analysis of variance (ANOVA). The triacylglycerol, apo B-48, and apo B-100 responses and the lipid and apolipoprotein compositions of the Sf > 400, Sf 60–400, and Sf 20–60 lipoprotein fractions after the 3 test meals were analyzed by two-factor repeated-measures ANOVA with interaction. A Bonferroni correction was used for the post hoc detection of significant pairwise differences. The data were checked for normality and were log transformed where necessary to render their distribution normal before statistical analysis. P values < 0.05 were taken as significant.

RESULTS

Triacylglycerol and apo B responses in the Sf > 400, Sf 60–400, and Sf 20–60 fractions

Fasting triacylglycerol, apo B-48, and apo B-100 concentrations in the Sf > 400, Sf 60–400, and Sf 20–60 fractions were not significantly different among study days and are shown in Tables 3 and 4. The triacylglycerol responses in the Sf > 400, Sf 60–400, and Sf 20–60 fractions after the SFA, PUFA, and MUFA meals are shown in Figure 1. In the Sf > 400 fraction, the PUFA meal resulted in a significantly lower AUC and IAUC than did the SFA or MUFA meal (P < 0.009; Table 3). Although there were no significant differences in the incremental triacylglycerol responses in the Sf 20–60 fraction, the AUC after the SFA meal was significantly greater than that after the PUFA meal (P = 0.02; Table 3). In both the Sf > 400 and Sf 60–400 fractions, there were significant differences in the patterns of lipemic response, with the PUFA meal showing a biphasic response compared with the MUFA meal (P = 0.001; Figure 1).

After the test meals, apo B-48 concentrations rose in the Sf > 400 fraction, with the MUFA meal resulting in significantly higher apo B-48 concentrations than did the PUFA meal (P = 0.02) and a different pattern of response than the SFA and PUFA meals (P = 0.001; Figure 2A). The MUFA meal also showed a significantly higher AUC compared with the PUFA meal (P = 0.005) and significantly higher IAUC compared with the SFA and PUFA meals (P < 0.02; Table 4). In the Sf 60–400 fraction, the apo B-48 IAUC for the PUFA meal was significantly lower than that for the MUFA meal (P = 0.01). There was a significant difference in the patterns of apo B-48 response between the test meals, with the MUFA meal showing a different pattern of response than the SFA and PUFA meals (P = 0.001; Figure 2B).

There were no significant differences in the Sf 20–60 apo B-48 responses after the SFA, PUFA, and MUFA meals (Figure 2C).

There were no significant differences in apo B-100 responses between the SFA, PUFA, and MUFA meals in the Sf > 400 and Sf 60–400 fractions, and the rise in apo B-100 concentrations was negligible in the Sf > 400 fraction compared with the Sf 60–400 and Sf 20–60 fractions (Figure 2). In the Sf 20–60
TABLE 3
Summary measures for fasting and postprandial triacylglycerol responses in plasma fractions with Svedberg flotation rates ($S_f$) > 400, 60–400, and 20–60 after meals enriched with different fatty acids$^1$

<table>
<thead>
<tr>
<th>Fraction</th>
<th>SFA meal</th>
<th>PUFA meal</th>
<th>MUFA meal</th>
<th>$P$ for ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_f &gt; 400$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting ($\mu$mol/L)</td>
<td>8.0 ± 2.5</td>
<td>11.2 ± 5.0</td>
<td>5.4 ± 1.3</td>
<td>NS</td>
</tr>
<tr>
<td>AUC ($\mu$mol - 480 min/L)</td>
<td>135.6 ± 16.9$^2$</td>
<td>76.5 ± 11.5</td>
<td>127.8 ± 21.2$^2$</td>
<td>0.002</td>
</tr>
<tr>
<td>IAUC ($\mu$mol - 480 min/L)</td>
<td>131.7 ± 16.4$^2$</td>
<td>71.1 ± 10.7</td>
<td>125.2 ± 21.1$^2$</td>
<td>0.001</td>
</tr>
<tr>
<td>$S_f$ 60–400</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting ($\mu$mol/L)</td>
<td>581.9 ± 142.4</td>
<td>441.5 ± 72.6</td>
<td>462.7 ± 84.2</td>
<td>NS</td>
</tr>
<tr>
<td>AUC ($\mu$mol - 480 min/L)</td>
<td>345.3 ± 81.5</td>
<td>218.1 ± 32.8</td>
<td>279.4 ± 45.6</td>
<td>NS</td>
</tr>
<tr>
<td>IAUC ($\mu$mol - 480 min/L)</td>
<td>66.0 ± 18.8</td>
<td>6.2 ± 25.6</td>
<td>57.3 ± 22.8</td>
<td>NS</td>
</tr>
<tr>
<td>$S_f$ 20–60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting ($\mu$mol/L)</td>
<td>238.2 ± 27.4</td>
<td>183.3 ± 26.0</td>
<td>201.1 ± 25.3</td>
<td>NS</td>
</tr>
<tr>
<td>AUC ($\mu$mol - 480 min/L)</td>
<td>122.5 ± 14.8$^2$</td>
<td>96.6 ± 10.9</td>
<td>109.6 ± 10.1</td>
<td>0.047</td>
</tr>
<tr>
<td>IAUC ($\mu$mol - 480 min/L)</td>
<td>8.2 ± 5.9</td>
<td>8.6 ± 6.0</td>
<td>13.0 ± 11.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

$^1$ All values are $\bar{x}$ ± SEM; $n = 10$. SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; AUC, area under the curve; IAUC, incremental area under the curve.

$^2$ Significantly different from the PUFA meal (one-way repeated-measures ANOVA followed by Student’s $t$ test with Bonferroni correction); $^3P < 0.001$, $^4P < 0.05$.

fraction, the AUC for the apo B-100 response after the SFA meal was significantly higher than that after the PUFA meal ($P = 0.02$; Table 4).

Apolipoprotein composition of fasting and postprandial $S_f > 400$, $S_f$ 60–400, and $S_f$ 20–60 apo B–containing particles

The apolipoprotein contents of the triacylglycerol-rich lipoproteins released after the SFA meal also contained significantly more apo E ($P < 0.05$; Figure 3), although there were no significant differences in apo C-II contents. However, there was a marked difference in the apo C-III contents of the triacylglycerol-rich lipoproteins after the test meals, with the SFA-rich lipoproteins containing significantly more apo C-III per particle than did the MUFA- and PUFA-rich lipoproteins ($P < 0.005$; Figure 3). Although the MUFA- and PUFA-rich lipoproteins showed similar apo C-III contents, the pattern of postprandial apo C-III enrichment of the lipoproteins was significantly different ($P = 0.009$). The triacylglycerol-rich lipoproteins released after the SFA meal also contained significantly more apo E ($\approx 5$-fold) and exhibited a different pattern of postprandial apo E enrichment than did the lipoproteins released after the MUFA and PUFA meals ($P < 0.001$; Figure 3).

TABLE 4
Summary measures for fasting and postprandial apolipoprotein B-48 and B-100 responses in plasma fractions with Svedberg flotation rates ($S_f$) > 400, 60–400, and 20–60 after meals enriched with different fatty acids$^1$

<table>
<thead>
<tr>
<th>Fraction</th>
<th>SFA meal</th>
<th>PUFA meal</th>
<th>MUFA meal</th>
<th>SFA meal</th>
<th>PUFA meal</th>
<th>MUFA meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_f &gt; 400$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting (nmol/L)</td>
<td>3.3 ± 0.3</td>
<td>3.8 ± 0.7</td>
<td>2.8 ± 0.4</td>
<td>0.04 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>AUC (nmol - 480 min/L)</td>
<td>3782 ± 428</td>
<td>3693 ± 714$^2$</td>
<td>5773 ± 831</td>
<td>69.0 ± 19.4</td>
<td>39.4 ± 6.1</td>
<td>57.1 ± 11.0</td>
</tr>
<tr>
<td>IAUC (nmol - 480 min/L)</td>
<td>2177 ± 364$^2$</td>
<td>1878.0 ± 492$^2$</td>
<td>4433 ± 753</td>
<td>51.8 ± 14.3</td>
<td>29.1 ± 5.4</td>
<td>45.0 ± 9.8</td>
</tr>
<tr>
<td>$S_f$ 60–400</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting (nmol/L)</td>
<td>7.7 ± 1.0</td>
<td>9.2 ± 1.6</td>
<td>12.1 ± 3.5</td>
<td>36.8 ± 8.3</td>
<td>29.6 ± 4.8</td>
<td>30.0 ± 4.3</td>
</tr>
<tr>
<td>AUC (nmol - 480 min/L)</td>
<td>7124 ± 743</td>
<td>7043 ± 1113</td>
<td>13357 ± 2414</td>
<td>19368 ± 4353</td>
<td>13294 ± 1608</td>
<td>16263 ± 1846</td>
</tr>
<tr>
<td>IAUC (nmol - 480 min/L)</td>
<td>3440 ± 450</td>
<td>2618 ± 589$^2$</td>
<td>7505 ± 1983</td>
<td>1695 ± 1325</td>
<td>~892 ± 1792</td>
<td>1844 ± 1319</td>
</tr>
<tr>
<td>$S_f$ 20–60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting (nmol/L)</td>
<td>11.4 ± 1.7</td>
<td>12.3 ± 2.1</td>
<td>10.5 ± 0.9</td>
<td>36.8 ± 5.4</td>
<td>27.8 ± 3.6</td>
<td>31.8 ± 3.2</td>
</tr>
<tr>
<td>AUC (nmol - 480 min/L)</td>
<td>6443 ± 616</td>
<td>7659 ± 1159</td>
<td>7622 ± 546</td>
<td>17967 ± 2478$^4$</td>
<td>12930 ± 1568</td>
<td>16766 ± 1480$^4$</td>
</tr>
<tr>
<td>IAUC (nmol - 480 min/L)</td>
<td>983 ± 424</td>
<td>1765 ± 532</td>
<td>2585 ± 417</td>
<td>292 ± 760</td>
<td>~398 ± 855</td>
<td>1495 ± 1296</td>
</tr>
</tbody>
</table>

$^1$ All values are $\bar{x}$ ± SEM; $n = 10$. SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; AUC, area under the curve; IAUC, incremental area under the curve.

$^2$ Significantly different from the MUFA meal (one-way repeated-measures ANOVA followed by Student’s $t$ test with Bonferroni correction) $^3P < 0.01$, $^4P < 0.05$.

$^3$ Significantly different from the PUFA meal, $P < 0.05$ (one-way repeated-measures ANOVA followed by Student’s $t$ test with Bonferroni correction).
The postprandial apo C-II and C-III contents of the Sf 60–400 lipoproteins were significantly different, with the triacylglycerol-rich particles released after the SFA meal containing more apo C-II and C-III than did the particles released after the MUFA meal (P = 0.005; Figure 3). The triacylglycerol-rich lipoproteins released after the SFA meal contained significantly more apo E than did the lipoproteins released after the MUFA and PUFA meals (P < 0.005). The pattern of postprandial apo E enrichment was also significantly different compared with the MUFA meal (P = 0.001; Figure 3); apo E content gradually increased with time postprandially for the SFA meal, whereas the apo E content showed a gradual decline with time for the MUFA meal.

**Sf 20–60 Fraction**

There were no significant differences in apo C-II, C-III, or E contents of the lipoproteins contained within the Sf 20–60 fraction after the SFA, PUFA, and MUFA meals. Although the lipoproteins released after the MUFA and SFA meals contained similar amounts of apo E, the pattern of the postprandial apo E enrichment of the particles was significantly different after the SFA meal (P = 0.007; Figure 3), with the enrichment of these particles with apo E increasing with time compared with a gradual decline in enrichment after the PUFA and MUFA meals.

### Lipid composition of fasting and postprandial triacylglycerol-rich lipoproteins

The lipid contents of the triacylglycerol-rich lipoproteins within the Sf > 400, Sf 60–400, and Sf 20–60 fractions after the SFA, PUFA, and MUFA meals are shown in Figure 4. The fasting triacylglycerol and cholesterol contents of the lipoproteins within the different fractions were not significantly different among postprandial days.

**Sf > 400 Fraction**

The postprandial triacylglycerol contents of the lipoproteins differed significantly between the meals, with the lipoproteins released after the SFA meal carrying significantly more triacylglycerol (∼1.5–2-fold) than those released after the MUFA meal (P = 0.01; Figure 4). The cholesterol content of the lipoproteins was also significantly different after the test meals; the lipoproteins released after the SFA meal contained significantly more cholesterol than did the lipoproteins released after the PUFA meal (P = 0.018) and had a significantly different pattern of postprandial cholesterol enrichment than did the lipoproteins released after the MUFA meal (P = 0.002; Figure 4).

**Sf 60–400 Fraction**

The postprandial triacylglycerol contents of the lipoproteins released after the SFA meal were significantly higher than the content of the lipoproteins released after the MUFA meal (P = 0.002; Figure 4). There were no significant differences in the cholesterol contents of the lipoproteins released after the SFA, PUFA, and MUFA meals, although the SFA meal showed a tendency for higher postprandial cholesterol contents (Figure 4).

**Sf 20–60 Fraction**

There was no significant effect of meal fatty acid composition on the postprandial triacylglycerol and cholesterol contents of the Sf 20–60 lipoproteins (Figure 4).

### DISCUSSION

The present study reports marked qualitative and quantitative differences in lipid and apolipoprotein responses to meals of different fatty acid composition that, we suggest, may be important in determining differences in the metabolic processing and subsequent atherogenicity of lipoproteins formed during the postprandial state. The triacylglycerol responses in both the Sf > 400 and Sf 60–400 fractions were similar for the SFA and MUFA meals.
meals, whereas responses to PUFA meals were lower. Estimation of the triacylglycerol:apo B ratios for the 2 fractions showed the ratios to be similar for the MUFA and PUFA meals but to be higher for the SFA meal, indicating triacylglycerol-rich lipoproteins of larger particle size. Most notably, the SFA meal resulted in Sf/L50140 400 and Sf 60–400 particles that contained significantly more triacylglycerol, apo C-III, and apo E than did particles formed after the MUFA meal and more apo C-III (Sf > 400 fraction only) and apo E compared with particles formed after the PUFA meal. These data suggest that although the MUFA and PUFA meals generated different numbers of triacylglycerol-rich lipoprotein particles (being considerably greater in response to the MUFA meal), the compositional characteristics of the circulating triacylglycerol-rich lipoproteins formed were similar. Conversely, the number of particles generated in response to the SFA and PUFA meals was similar, although the composition of the particles differed. In particular, the particles formed after the SFA meal were markedly enriched with apo E; in the Sf > 400 fraction, enrichment with apo E was 4-fold higher, and in the Sf 60–400 fraction enrichment was 2-fold higher, after the SFA than after the MUFA or PUFA meal.

**FIGURE 2.** Mean (±SEM) apolipoprotein (apo) B-48 and apo B-100 responses in plasma fractions with Svedberg flotation rates (Sf) >400 (A), Sf 60–400 (B), and Sf 20–60 (C) after test meals enriched in saturated fatty acids (●), polyunsaturated fatty acids (○), or monounsaturated fatty acids (■). Values are for 10 middle-aged men. The y axes for the apo B-48 and apo B-100 concentrations in the Sf > 400 and Sf 20–60 fractions differ. For apo B-48 responses in the Sf > 400 and Sf 60–400 fractions, there was a significant main effect of meal fat type (P < 0.05), a significant time effect (P < 0.001), and a significant meal × time interaction (P = 0.001).
Few previous studies have compared postprandial apolipoprotein contents of triacylglycerol-rich lipoproteins after meals of different fatty acid composition. Consistent with the present study, the study by Brouwer et al (20) showed no apparent effects of PUFA- and MUFA-enriched emulsions on apo C-II content, and the study by Mero et al (21) showed that compared with PUFA, a SFA-rich mixed meal showed a nonsignificant tendency for higher apo E concentrations in the S_f > 400 fraction postprandially. Interestingly, the other SFA-rich meal (cream) in that study resulted in lower apo E concentrations 6–8 h postprandially than after the mixed SFA-rich meal. This finding suggests that the vehicle used to introduce the fat into the test
meal, ie, mixed food versus oils, influences the metabolism of the triacylglycerol-rich lipoproteins in the enterocyte or circulation. Other authors have shown differences in the postprandial triacylglycerol response when dairy fats of identical fat composition but provided in different food matrices are given (30). The use of a single fat source as opposed to combinations of SFA-, PUFA-, and MUFA-rich foods in the test meals of the present study may underlie our findings and may also explain the differences in the patterns of the $S_f > 400, S_f 60–400$, and $S_f 20–60$ fractions. For the changes in TG and cholesterol contents in the $S_f > 400$ fraction, there was a significant time effect ($P < 0.001$) and a significant meal $\times$ time interaction ($P < 0.03$). In the $S_f 60–400$ fraction, there was a significant meal $\times$ time interaction ($P = 0.001$) and a significant main effect of meal fat type ($P = 0.005$) for TG.

Greater numbers of large ($S_f > 400$) and moderately sized ($S_f 60–400$) triacylglycerol-rich lipoproteins. We have proposed that this response reflects a greater capacity to promote chylomicron formation and secretion from the enterocyte after exposure to MUFAs, a proposition that is supported by studies using the Caco-2 cell model (36–41).

A greater number of circulating triacylglycerol-rich lipoprotein particles might be considered to be an adverse postprandial response and appears to conflict with the widely reported cardioprotective effects of MUFA-rich diets and meals. However, a notable feature of the triacylglycerol and apo B-48 postprandial profiles for the MUFA meals is the rapidly declining concentrations result in the return of the particle number to fasting levels. This suggests that, whatever the origin of the greater rise in particle number, the particles do not persist within the circulation and are therefore less likely to have adverse atherogenic consequences. The marked decline in apo C-III content in the late postprandial period for the MUFA meal may also be relevant, because apo C-III inhibits remnant removal. The major compositional differences in triacylglycerol-rich lipoproteins observed in response to SFA-rich meals raise important questions as to the origin of these differences and their

**FIGURE 4.** Mean ($\pm$SEM) time response curves for changes in lipid contents [number of triacylglycerol (TG) or cholesterol molecules per apolipoprotein (apo) B particle] in plasma fractions with Svedberg flotation rates ($S_f$) $> 400$, $S_f 60–400$, and $S_f 20–60$ after test meals enriched in saturated fatty acids (●), polyunsaturated fatty acids (○), and monounsaturated fatty acids (■). Values are for 10 middle-aged men. The $y$ axes for the lipid contents may differ in the $S_f > 400$, $S_f 60–400$, and $S_f 20–60$ fractions. For the changes in TG and cholesterol contents in the $S_f > 400$ fraction, there was a significant time effect ($P < 0.001$) and a significant meal $\times$ time interaction ($P < 0.03$). In the $S_f 60–400$ fraction, there was a significant meal $\times$ time interaction ($P = 0.001$) and a significant main effect of meal fat type ($P = 0.005$) for TG.
consequences for the metabolism and subsequent atherogenicity of the apolipoprotein-rich particles. Detailed studies in animals have shown that meal fatty acid composition influences the metabolism of the triacylglycerol-rich lipoproteins in the circulation and their subsequent uptake by the liver. The rate of hydrolysis of the particles by LPL and HL and internalization of the particles are influenced by fatty acid composition, with PUFA-rich particles being more rapidly hydrolyzed and MUFAs and PUFA remnants being more rapidly internalized by the liver than are SFA-rich particles (7, 8, 42). It is generally assumed that these metabolic differences relate to differences in the lipolytic activity of LPL against particles of different fatty acid composition. We suggest that our present findings indicate that at least some of the differences in particle composition and lipid content could be secondary to differences in the apolipoprotein composition of the S; > 400 and S; 60–400 lipoproteins.

The factors that influence the amounts of the different apolipoproteins that are incorporated into triacylglycerol-rich lipoproteins have not been fully elucidated. A recent study by Kovar and Havel (43) suggested that particle surface area is a major determinant of the content of apolipoproteins on triacylglycerol-rich lipoproteins. Of relevance to the findings of the present study, work using in vitro triacylglycerol emulsions showed that the binding densities of apo C-II, C-III, and E were greater for larger lipid emulsions than for smaller ones (44). Taking account of previous work showing greater hydrolysis of particles containing unsaturated fatty acids, we suggest that slower initial rates of hydrolysis of SFA-rich particles may lead to greater accumulation of apo C-II, C-III, and E, and that the accumulation of apo C-III prevents the rapid clearance of the SFA-rich particles by the liver.

In the present study, triacylglycerol:apo B ratios were highest for the SFA meal and less for the MUFA and PUFA meals, which suggests that in the later postprandial period, the SFA particles were larger than the MUFAs- or PUFA-rich particles. However, we have only been able to estimate the size of the particles within the circulation, and this may not be the most relevant measure. The size of the nascent chylomicrons in the intestinal lymph may be more important for the acquisition of apolipoproteins, and this may be the most important determinant of their apolipoprotein composition. The increased apo E content of the large triacylglycerol-rich lipoproteins after the SFA meal would normally indicate that such particles would be rapidly cleared from the circulation (45). However, these particles also show greater apo C-III content, which would impair the hydrolysis of particles by LPL and HL and subsequent particle uptake by the liver. This apolipoprotein has also been shown to increase the activity of the cholesterol ester transfer protein, and the accumulation of the SFA-enriched particles may affect the metabolism and atherogenicity of the S; 60–400 and S; 20–60 fraction lipoproteins.

In conclusion, our study of the effect of meal fatty acids on the lipid and apolipoprotein composition of triacylglycerol-rich lipoprotein fractions has generated novel findings. In this group of middle-aged men, we found that the ingestion of an SFA-enriched meal markedly increases the triacylglycerol, cholesterol, apo C-III, and apo E content of large (S; > 400) and the apo E content of moderately sized (S; 60–400) triacylglycerol-rich lipoproteins compared with PUFA and MUFA meals. We suggest that these compositional differences may have adverse atherogenic consequences that are additional to those resulting from the LDL-raising effects of SFAs. Second, the magnitude of the apo B-48 responses in the S; > 400 and S; 60–400 fractions were influenced by the meal fatty acids, with a more marked accumulation and rapid decline in particle number for the MUFA meal. We suggest that the rapid removal of MUFA-rich particles indicated by the rapid change in apo B-48 and apo C-III concentrations in the declining part of the postprandial response offers antiatherogenic benefits additional to the LDL-reducing effects of MUFAs.

This study was designed by CMW, PY, and KGJ, and the data were collected and analyzed by EJW, PAB, and KGJ. The manuscript was written by KGJ and CMW. The authors had no conflict of interests.

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Temporal pattern of de novo lipogenesis in the postprandial state in healthy men

Maureen T Timlin and Elizabeth J Parks

ABSTRACT
Background: Recent data suggest that hepatic de novo lipogenesis (DNL) is elevated in the fed state compared with the fasting state, but the rate at which lipogenesis can increase with meal consumption is currently unknown.

Objective: The objective was to quantify the diurnal pattern of lipogenesis after 2 consecutive mixed meals were fed to healthy men (n = 8).

Design: A liquid diet was administered after a 12-h fast. During the fasting and postprandial periods, serum insulin, glucose, triacylglycerol, and nonesterified fatty acid concentrations were measured, and rates of DNL were quantified via intravenous infusion of [1-13C] sodium acetate and mass isotopomer distribution analysis.

Results: The temporal pattern of postprandial lipogenesis was similar in all subjects. Lipogenesis rose significantly from 4.7 ± 3.3% at fasting, peaked at 18.2 ± 7.1% after meal 1 (P = 0.003 compared with fasting), rose further to 23.1 ± 8.9% after meal 2 (P = 0.01 for difference between meals), and then decreased toward baseline (P < 0.001). Lipogenesis peaked 4.2 h after the meals; lipoprotein-triacylglycerol concentrations peaked sooner, 2.0 h after the meals (P < 0.02). Maximum postprandial DNL ranged from 10.3% to 37.5%. Peak insulin concentrations after meal 1 correlated with peak DNL (R = 0.838, P = 0.037), although the leanest subjects had some of the highest rates of postprandial DNL.

Conclusion: These data confirm the acute stimulation of DNL after meals in healthy subjects and validate the contribution of this pathway to elevations in triacylglycerol concentration. Am J Clin Nutr 2005;81:35–42.

KEY WORDS De novo lipogenesis, hepatic lipogenesis, triacylglycerol

INTRODUCTION
Several epidemiologic studies have shown that elevated lipids in the postprandial state pose an individual risk for the development of coronary artery disease (1–3). Clinical studies have also shown that both the concentration and duration of postprandial lipemia play a role in the development and progression of atherosclerosis (4–6). In the postprandial state, intestinally derived lipoproteins, chylomicrons, are responsible for the transportation of dietary triacylglycerol in blood. However, after a meal, dietary carbohydrate can be made into fatty acids in the liver (7) via a process called de novo lipogenesis (DNL). These newly made fatty acids are found in heptatically derived VLDLs. VLDLs are present in blood in both the fasting and fed states, but the sources of fatty acids available for VLDL-triacylglycerol synthesis may change after a meal. One potentially important source of fatty acids in VLDLs is the pathway of DNL. The rate of increase in lipogenesis after a meal is of interest because this rate may be a key factor influencing the atherogenicity of the postprandial state. Elevated lipogenesis has been shown to increase the percentage of saturated fatty acids in VLDL-triacylglycerol, which could increase the risk of thrombosis (8) and potentially affect membrane receptor function (9). Furthermore, newly made fatty acids may add to the triacylglycerol content of VLDL particles, thereby increasing absolute triacylglycerol secretion rates (10, 11), or the lipogenic rate may provide a signal in the hepatocyte to increase the esterification of fatty acids from other sources [eg, the plasma nonesterified fatty acid (NEFA) pool].

Previous studies in humans have shown that chronic consumption of diets high in carbohydrate (>50% from mono- and disaccharides) results in a greater amount of newly made fatty acids detected in the blood in fasting persons (12–15). Data are available to document DNL in the fed state; however, the acute effect of food intake immediately after food consumption has not been assessed. Two previous studies measured fed-state lipogenesis after chronic consumption (5–25 d) of high-carbohydrate diets (12, 16). Although average fed-state data were calculated from a selected number of measurements collected on the last day of each study, a pattern of postprandial DNL could not be determined. Another recent study showed higher rates of lipogenesis in obese persons than in lean healthy persons, in both the fasting and fed states, after consumption of a carbohydrate-rich bolus (17). However, fed-state lipogenesis was assessed after only one meal was consumed (80% of energy from carbohydrate) and after only a limited number of postprandial data points were taken, which made it difficult to detect the minute-by-minute pattern of postprandial DNL. Therefore, the immediate effect of eating a mixed meal to stimulate DNL has not been studied. The goals of the present study were two-fold: 1) to document the pattern of postprandial lipogenesis when healthy humans consumed a liquid meal rich in glucose; and 2) to quantify a diurnal pattern of lipogenesis after 2 consecutive meals. Our hypothesis was that...
lipogenesis would increase acutely after food consumption, but the magnitude of this effect was unknown. To achieve these goals, liquid meals high in monosaccharides were fed.

SUBJECTS AND METHODS

Human subjects

Subject recruitment occurred via advertisement, and written informed consent was obtained (University of Minnesota, IRB 0106M01641). Screening was conducted at the General Clinical Research Center (GCRC) at Fairview University Medical Center and consisted of 2 fasting blood draws to verify hematocrit, hemoglobin, fasting triacylglycerol, glucose, and insulin concentrations. Before the blood draw, subjects had fasted for 12 h, were well-hydrated, and had abstained from alcohol for ≥48 h. Body composition was determined by dual-energy X-ray absorptiometry (Lunar Corp, Madison, WI). Our original intent was to study both men and women in the immediate postprandial period. By chance, men were the first 3 subjects to be entered into the protocol; in these initial studies, intersubject variability appeared of sufficient magnitude to warrant restriction of subject recruitment to a single sex. To be eligible for the study, subjects had to be aged 20–55 y, be nonsmokers, and have a stable body weight. The subjects maintained consistent exercise and activity patterns for the 3 mo previous to the metabolic study. Subjects were excluded if they had a history of diabetes or any other metabolic disease or if they were taking medication known to affect lipid metabolism.

Study design

Each subject completed a single 24-h inpatient study. For 3 d before this metabolic study, the subject consumed a weight-maintaining diet that provided a constant energy intake based on the Harris-Benedict equation (18) and 3-d food records of usual intake. Because the composition of the diet can significantly affect fatty acid metabolism (19), the goal of the prestudy diet was to provide 55% of total energy from carbohydrate, 30% from fat, and 15% from protein. The actual average profile of the 3-d intake. Because the composition of the diet can significantly affect fatty acid metabolism (19), the goal of the prestudy diet was to provide 55% of total energy from carbohydrate, 30% from fat, and 15% from protein. The actual average profile of the 3-d prestudy diet was as follows: 11840 ± 1590 kJ (51 ± 1% from carbohydrate, 34 ± 0.5% from fat, and 15 ± 1% from protein), 32 ± 9 g fiber, and 7 ± 2 g n–3 fatty acids. Under the direction of a registered dietitian, the diets were prepared by the subjects themselves and consisted of whole foods. On day 1 of the inpatient study, the subject reported to the GCRC between 1630 and 1700. At 1730, 1 intravenous line was placed in the antecubital vein of each arm: one for the administration of stable isotope and meal 1 in 25 min and meal 2 in 26.5 min. Indirect calorimetry was performed for 30 min from 0630 to 0700 (fasting state) and from 1530 to 1600 (fed state) on day 2 with a Deltatrac II Metabolic Cart (Sensor Medix, Yorba Linda, CA) in the hooded mode. The subjects rested, watched television, or read during the infusion study. Nonenergy-containing, noncaffeinated drinks were available on request.

Metabolic infusion protocol

In all subjects, an intravenous infusion containing sodium [1-13C]acetate (isotopic purity >98%; Cambridge Isotope Laboratories, Andover, MD) was started at 1730 on day 1 and ran until 1800 on day 2. Starting at 0000 and continuing to 1800 on day 2, frequent blood samples were drawn into tubes containing 1 mg EDTA/mL and plasma was separated immediately by centrifugation (3000 rpm, 1500 × g, 10 min, 10 °C). The samples were kept on ice while EDTA, benzamidine, gentamicin sulfate, chloramphenicol, trolox, and phenylmethylsulfonyl fluoride were added as a preservative cocktail (20) and were then portioned for glucose, insulin, and serum triacylglycerol analysis. Plasma samples for nonesterified fatty acid (NEFA) analysis were extracted immediately with a 30:70 heptane/isopropanol mixture containing 10 μL undecanionic acid (11:0) or pentadecanonic acid (15:0) as a fatty acid internal standard (Sigma Chemical Co, St Louis).

Lipoprotein isolation

Lipoprotein isolation and subfractionation of total triacylglycerol-rich lipoproteins (tTRLs), the fraction with a Svedberg flotation unit (Sf) >400 (>400Sf), and the fraction with an Sf of 60–400 (60–400Sf) were performed as described previously (21). Briefly, tTRLs represent all particles with a density <1.0063; the >400Sf fraction contains particles >70 nm in diameter and the 60–400Sf fraction contains particles 40–70 nm in diameter. This method isolates the largest most buoyant particles with a very short half-life and, thus, yields lipogenesis data that reflect the most recently secreted particles by the liver. After separation of the tTRL, >400Sf, and 60–400Sf fractions, fasting and fed apolipoprotein (apo) B-48 and apo B-100 concentrations of each lipoprotein fraction were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis to confirm purity of the 60–400Sf fraction (20). Plasma NEFAs and triacylglycerols were separated from the other lipids (phospholipid, cholesterol ester, and free cholesterol) via chromatography and were derivatized to their methyl esters as described previously (21).
Gas chromatography–mass spectrometry and analysis of metabolic variables

The methyl esters of plasma NEFAs and tTRLs (the $>400_{4f}$ and 60–400Sf fractions) were separated on a Quadrex 007–23 fused silica capillary column, 50 m $\times$ 0.25 mm internal diameter $\times$ 0.25 $\mu$m film thickness (Quadrex Corp, New Haven, CT). Fatty acid composition was measured by flame ionization detection (22). Gas chromatography (GC) was performed on a Hewlett-Packard 5890 instrument (Hewlett-Packard, Norwalk, CT) fitted with a 7683 automatic split injection system and a flame ionization detector. Mass spectrometry (MS) was performed with the use of an HP-1 column (25 m, 250 $\mu$m inner diameter $\times$ 0.33 $\mu$m film thickness) in a Hewlett-Packard 6890 GC (Hewlett-Packard, Eagan, MN) with helium as the carrier gas. Selected ion monitoring was used for ions with mass to charge ratios (m/z) of 270, 271, and 272, which were analyzed with an HP 5973 MS fitted with an ETP electron multiplier (SGE Incorp, Austin, TX). Comparable ion peak areas between an unlabeled standard and biological samples were achieved by either adjusting the volume injected, diluting, or concentrating the sample when needed. Newly made fatty acids from DNL were calculated by mass isotope distribution analysis (23). Concentrations of glucose in plasma samples were measured with a Vitros Analyzer 950 (Ortho-Clinical Diagnostics, Rochester, NY) and concentrations of insulin were determined via chemiluminescent immunoassay (Diagnostic Products Corporation, Los Angeles). Serum triacylglycerol and NEFA concentrations were determined via enzymatic assay (Wako Chemicals USA Inc, Richmond, VA) with a microtiter spectrophotometer (model EL 340 Microplate; Bio-Tek Instruments Inc, Winooski, VT).

Calculations and statistical analysis

Individual data from each subject were plotted and evaluated qualitatively, and the general pattern was described. Values for the metabolic variables (eg, triacylglycerol and glucose) attained over the 11-h feeding period were analyzed quantitatively to obtain averages, time to peak height, and rate of increases or decreases (slope), etc. Statistical analyses were performed with the use of STATVIEW for WINDOWS (version 5.0.1; SAS Institute Inc, Berkeley, CA). Differences between the fasting- and fed-state data were analyzed with the use of paired Student’s t test. Correlations were analyzed by using simple regression, and the effects of time were analyzed with the use of multiple regression.

RESULTS

Baseline subject characteristics are presented in Table 1 and reflect healthy body weights and normal fasting plasma insulin, glucose, and triacylglycerol concentrations at screening. The changes in insulin, glucose, triacylglycerol, and NEFA concentrations between fasting and feeding are shown in Table 2. As expected, glucose and insulin concentrations averaged over the fasting and the fed states were higher with feeding. Peak concentrations of insulin reached after each meal indicated a significantly greater response after meal 1 (759.3 $\pm$ 378.0 pmol/L) than after meal 2 (437.7 $\pm$ 182.8 pmol/L; $P = 0.006$). No significant difference between the 2 meals was found for peak concentrations of glucose. Average concentrations of triacylglycerol in all lipoprotein fractions were higher with feeding ($P < 0.03$). Peak triacylglycerol concentrations in the tTRL also tended to be higher after meal 2 than after meal 1 ($P = 0.066$). For the $>400_{4f}$ fraction, no significant difference between meal peaks was found; the same was true for the 60–400Sf fraction. The concentration of NEFAs tended to drop from the fasting to the fed states, with greater suppression after meal 1 (nadir: 0.14 $\pm$ 0.03 mmol/L) than after meal 2 (0.17 $\pm$ 0.08 mmol/L). Energy expenditure increased with feeding from 4.60 $\pm$ 0.59 kJ $\cdot$ kg$^{-1} \cdot$ min$^{-1}$ in the fasting state to 5.23 $\pm$ 1.00 kJ $\cdot$ kg$^{-1} \cdot$ min$^{-1}$ in the fed state ($P = 0.024$), as was expected. Fat oxidation was 0.62 $\pm$ 0.20 and 0.51 $\pm$ 0.39 mg $\cdot$ kg$^{-1} \cdot$ min$^{-1}$ in the fasting state and in the fed state ($P = 0.340$) and glucose oxidation was 0.85 $\pm$ 0.51 and 1.16 $\pm$ 0.80 mg $\cdot$ kg$^{-1} \cdot$ min$^{-1}$ ($P = 0.214$) in the fasting and fed states, respectively.

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
</tr>
<tr>
<td>Age (y)</td>
</tr>
<tr>
<td>Weight (kg)</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
</tr>
<tr>
<td>Body fat (kg)</td>
</tr>
<tr>
<td>Body fat (%)</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
</tr>
<tr>
<td>Lean mass (%)</td>
</tr>
<tr>
<td>Fasting insulin (pmol/L)</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
</tr>
<tr>
<td>Fasting triacylglycerol (mmol/L)</td>
</tr>
</tbody>
</table>

$^1$ All values are $\bar{x}$ $\pm$ SD from screening visits; range in parentheses.

De novo lipogenesis

Individual data for each subject are shown in Figure 1. In the first 2 subjects studied, only tTRLs were isolated. Subject 1 (Figure 1A) had a minimal change in DNL after meal 1, and lipogenesis reached a peak value of only 3.9% 4 h after meal 2. Subject 2 (Figure 1B) had higher DNL throughout the night (note the different scales on the y axis between the subjects) and had higher fed levels of DNL, with fed-state peak values reaching 28.8% 4 h after meal 1 and 29.9% 3 h after meal 2. It was possible that the observed difference in lipogenic rates between these 2 subjects could have been due to a difference in the quantity of remnants present in the tTRL fractions. To remove this potential confounding effect, tTRL particles underwent an additional fractionation step for the remainder of the study, and lipogenesis was measured in the 60–400Sf fraction because this procedure isolates a purer VLDL fraction (which reflects hepatic lipogenic rates). Lipogenesis was also measured in the $>400_{4f}$ fraction (a combined fraction of chylomicrons and large VLDLs). The lipogenesis data for 60–400Sf fraction only in the remaining 6 subjects are presented individually in Figure 1, C–H, and mean data for all fractions are shown in Figure 2. Although the general pattern of DNL was not different between subjects (Figure 1, C–H), the maximal level of lipogenesis achieved varied between subjects, ranging from 10.3% to 29.8% after meal 1 and from 14.1% to 37.5% after meal 2. Toward the end of the metabolic test, DNL was decreasing in all but one subject (Figure 1E). Surprisingly, the 2 subjects with the lowest percentage body fat had the highest levels of fed-state DNL in the 60–400Sf fraction (Figure 1, B and F). At the time of the study, subject 6 was actively engaged in aerobic activity on a recreational basis 5 times/wk, which put him at the upper end of our study inclusion criteria. This anecdotal information suggests that his high fitness...
TABLE 2
Average fasting, fed, and peak values for insulin, glucose, triacylglycerol, total triacylglycerol-rich lipoprotein (tTRL), nonesterified fatty acids (NEFAs), and de novo lipogenesis (DNL)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fasting$^j$</th>
<th>Fed$^j$</th>
<th>$P$</th>
<th>Meal 1 peak$^j$</th>
<th>Meal 2 peak$^j$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (pmol/L)$^j$</td>
<td>31.0 ± 17.3$^4$</td>
<td>228.5 ± 139.2</td>
<td>0.0004</td>
<td>759.3 ± 378.0</td>
<td>437.7 ± 182.8</td>
<td>0.006</td>
</tr>
<tr>
<td>Glucose (mmol/L)$^j$</td>
<td>5.1 ± 0.3</td>
<td>5.8 ± 0.8</td>
<td></td>
<td>7.8 ± 1.1</td>
<td>7.5 ± 0.8</td>
<td>0.520</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/L)</td>
<td>tTRL$^L$</td>
<td>0.41 ± 0.37</td>
<td>0.002</td>
<td>0.68 ± 0.40</td>
<td>1.06 ± 0.61</td>
<td>0.066</td>
</tr>
<tr>
<td></td>
<td>&gt;400Sf$^d$</td>
<td>0.05 ± 0.05</td>
<td>0.013</td>
<td>0.2 ± 0.13</td>
<td>0.37 ± 0.29</td>
<td>0.176</td>
</tr>
<tr>
<td></td>
<td>60–400Sf$^d$</td>
<td>0.17 ± 0.16</td>
<td>0.032</td>
<td>0.35 ± 0.20</td>
<td>0.40 ± 0.24</td>
<td>0.357</td>
</tr>
<tr>
<td></td>
<td>NEFA (mmol/L)$^j$</td>
<td>0.49 ± 0.20</td>
<td>0.016</td>
<td>0.14 ± 0.03$^3$</td>
<td>0.17 ± 0.08$^5$</td>
<td>0.096</td>
</tr>
<tr>
<td>DNL (%)</td>
<td>tTRL$^L$</td>
<td>4.1 ± 3.8</td>
<td>0.001</td>
<td>14.8 ± 9.2</td>
<td>17.7 ± 8.9</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>&gt;400Sf$^d$</td>
<td>2.4 ± 1.6</td>
<td></td>
<td>6.4 ± 3.4</td>
<td>7.8 ± 3.7</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>60–400Sf$^d$</td>
<td>4.7 ± 3.3</td>
<td></td>
<td>18.2 ± 7.1</td>
<td>23.1 ± 8.9</td>
<td>0.013</td>
</tr>
</tbody>
</table>

$^j$ Values represent concentrations averaged over the time frame for the fasting (−3 to −1 h) and fed (0.25 to 11 h) states.

$^d$ Values are peaks unless noted otherwise. *Peak* refers to height achieved after each meal for insulin, glucose, triacylglycerol, and DNL relative to the fasting value.

$^L$ $n = 8$.

$^4$ ± SD (all such values).

$^3$ Values are nadirs. *Nadir* refers to the decrease in NEFAs occurring after meals relative to the fasting value.

level may have been associated with an increased capacity to make fat. The average values of all subjects showed that newly made triacylglycerol fatty acids in tTRLs and in the 60–400Sf fraction decreased significantly throughout the night (Figure 2; time effect: $P < 0.0001$). However, after consumption of meal 1, the rate of appearance of newly made fatty acids in the tTRL and the 60–400Sf fraction increased significantly from 1.5 to 4 h ($P < 0.0001$) and then began to decrease at 4.5 h. The pattern appeared similar but had a shorter initial lag period after meal 2, for which the rate of increase in tTRL and in the 60–400Sf fraction significantly increased from 1.5 to 3 h postmeal ($P < 0.0001$). During the last 3 h of the metabolic study, DNL decreased significantly in all fractions ($P < 0.0001$). As shown in Figure 2, the postprandial <60–400Sf fraction contained a larger percentage of newly made fatty acids than did the other 2 fractions. DNL in tTRLs and in the 60–400Sf fraction significantly increased from 1.5 to 3 h postmeal ($P < 0.0001$). After meal 1, peak insulin concentrations positively correlated with peak triacylglycerol concentration after meal 1 ($R = 0.949, P = 0.004$; data not shown). At its highest peak, DNL in the >400Sf fraction reached a value of only 7.2 ± 3.7%, whereas DNL in the 60–400Sf fraction peaked at 18.2 ± 7.2% after meal 1 and 23.1 ± 8.9% after meal 2. The temporal pattern of concentrations in the 60–400Sf triacylglycerols is shown in Figure 2. Newly made fatty acids were rising gradually as triacylglycerol concentration after meal 1 (1.3 compared with 2.7 h, respectively; $P = 0.016$; Figure 2).

Correlations
In the present study, the magnitude of the peak insulin concentration after meal 1 correlated with peak DNL in the 60–400Sf fraction ($R = 0.838, P = 0.037$). After meal 2, peak insulin concentrations positively correlated with peak triacylglycerol concentration in tTRLs ($R = 0.753$) and in the >400Sf fraction ($R = 0.915, P < 0.03$ for both); insulin failed to correlate with lipogenesis in any fraction after meal 2. Data from previous studies showed a positive correlation between an increase in fasting DNL in VLDLs associated with chronic consumption of high-carbohydrate diets and changes in fasting triacylglycerol concentrations (14, 15). Specifically, Schwarz et al (15) found that the increase in the fasting percentage of DNL correlated with the percentage increase in fasting triacylglycerol concentrations associated with 5 d of a high-carbohydrate diet ($R = 0.932, P < 0.05$). In the present study, a positive relation was also found between fasting DNL in the 60–400Sf fraction and the acute increase in triacylglycerol after meal 1 ($R = 0.860, P < 0.03$). These observations relate to the level of lipogenesis in the fasting state in these subjects and to changes in triacylglycerol concentration after the liquid meal. However, if lipogenesis contributes directly to increases in triacylglycerol concentrations postprandially, the acute change in DNL after a meal should correlate with the change in triacylglycerol concentration after that meal. Surprisingly, when this relation was tested in the present study, a negative correlation was found ($R = −0.888, P = 0.018$) (Figure 3).

DISCUSSION
The present study was conducted to document the temporal pattern of postprandial lipogenesis in healthy humans. Consistent with previous findings (14), lipogenesis was shown to decrease throughout the night, which is in line with a natural transition to the fasting state. In support of this pattern, the
FIGURE 1. De novo lipogenesis (DNL) in total triacylglycerol-rich lipoproteins (subjects 1 and 2) and in the triacylglycerol fraction with a Svedberg flotation unit of 60–400 (subjects 3–8) during the fasting period (between −8 and 0 h) and after the consumption of 2 meals at time points 0 and 5 h. The solid lines indicate the percentage of DNL; the dashed lines indicate insulin concentrations.
consumption of both eucaloric diets and excess carbohydrate-containing diets has shown lower DNL in the fasting than in the fed state (16). We fed a meal high in mono- and disaccharides, because this has been shown to elicit higher values of DNL (12, 13, 17). Ours is the first study to document the immediate effect of a mixed meal to stimulate lipogenesis in humans. Although many researchers who study fat synthesis in animal models would have predicted the higher postprandial DNL observed in the present study, both the timing and magnitude of this effect in humans were unknown. The novel contributions of this work are as follows.

First, DNL rose significantly after meal 1 and further increased after meal 2; the second peak was approximately 27% greater than the first peak. These observations more completely define the effect of meals originally suggested by the diurnal pattern of Hudgins et al (14), who found progressively higher daylong rates of lipogenesis based on data obtained intermittently over a 24-h period. Our data also expand on the data of Hellerstein et al (24), who fed healthy subjects either hourly boluses of liquid formula or a solid breakfast meal, and on the data of Marques-Lopes et al (17), who similarly showed minor increases in DNL after a carbohydrate-rich meal bolus. During feeding, lipogenesis rose from 1% (fasting) to approximately 5% (fed) in lean persons in both studies and from 3% to 9% in the obese subjects studied by Marques-Lopes. Both studies fed more carbohydrate (159-245 g) than that fed in the present study (125 g/bolus); however, we observed much higher rates of lipogenesis (23%). One likely reason for this discrepancy is because the acetate infusion in the present study began 14 h before the fed-state measurements were made, whereas these other studies infused acetate for 7–9 h before the first measurement. The present data support the concept that a longer duration of labeling is necessary to equilibrate newly made fatty acid pools in the liver (12). Another factor that contributed to the higher peak values observed in the present study was the method of lipid fractionation that we used (21). Without this procedure, most of the particles in the tTRL fraction would be VLDL remnants and intermediate-density lipoproteins, whose turnover is 6–8 h (25).

Second, as shown here, measurement of the temporal pattern showed that the largest absolute contribution of newly made fatty acids to VLDL-triacylglycerol occurred toward the end of the metabolic test (11 h), at which time triacylglycerol concentrations were decreasing faster than was lipogenesis (Figure 2). After both meals, postprandial triacylglycerol concentration peaked roughly between 2 and 3 h postmeal, a consistent finding in the literature (1, 5, 26–28), whereas peak DNL occurred slightly >4 h postmeal. This delayed pattern of DNL after meal 1 was consistent with the time delay for sensitization of the liver to up-regulate fatty acid synthase enzyme as a result of an early elevation in insulin concentration. In the present study, the change in insulin concentration postprandially correlated positively with the change in hepatic lipogenesis after meal 1. Furthermore, we found a negative correlation between the percentage increase in DNL and the absolute increase in triacylglycerol concentration (Figure 3). Thus, larger increases in postprandial triacylglycerol were associated with smaller increases in the percentage of newly made fatty acids in VLDL-triacylglycerol early in the postprandial period. Meal 2 resulted in much lower insulin peaks, as has been observed previously (29). Despite the lower insulin response after meal 2, the percentage of newly made fatty acids was 27% higher after meal 2. Interestingly, the significant association between insulin and DNL was lost after meal 2, as was the negative correlation between the change in triacylglycerol concentration and the change in DNL. This could have resulted from the presence of remnants, because observations from independent studies are beginning to support the concept that consumption of carbohydrate is associated with an accumulation of remnants in the plasma (21, 30). Because remnants contain substantial amounts of triacylglycerol but few, if any, newly made fatty acids (tTRL and >400Sf; Figure 2), they would dilute the apparent percentage of DNL in heparically derived particles. Lastly, although the general pattern of lipogenesis among the subjects was strikingly similar, close inspection of the curves raises critical questions as to the metabolic basis for the differences between subjects.

**FIGURE 2.** Mean (±SE) de novo lipogenesis (DNL) in total triacylglycerol-rich lipoprotein (tTRL) and in triacylglycerol fractions with a Svedberg flotation unit of 60–400 (60–400Sf) or >400 (>400Sf) during the fasting period (between −8 and 0 h) (n = 8) and in VLDL-triacylglycerol (n = 6) after the consumption of 2 meals.

**FIGURE 3.** Absolute change in triacylglycerol fractions with a Svedberg flotation unit of 60–400 (60–400Sf) from fasting to meal 1 compared with the change in de novo lipogenesis (DNL) in the 60–400Sf fraction from fasting to meal 1 (R = 0.888, P = 0.018).
Factors that can contribute to the variability in lipogenesis between subjects could include differences in the rate of digestion, the time taken to consume the liquid formula, other sources of fatty acids that can contribute to VLDL-triacylglycerol, and the relative caloric balance of the subjects before the metabolic test. Given that the meals were liquid formula, it is likely that the rate of digestion was faster than that with solid food. A fast rate of absorption would tend to increase the observed lipogenic rates and whether individual differences significantly impacted lipogenesis in the present study is unknown. Because the duration of meal consumption was small (≈25 min) relative to that in the 11-h study, it is unlikely that meal-duration differences between subjects contributed to the variability observed in lipogenesis (Figure 1). DNL is just one of many sources of fatty acids that could contribute to VLDL-triacylglycerol secreted by the liver postprandially. These fatty acid sources (eg, NEFAs, dietary lipid, and splanchnic pools) must all be regulated, and their fluxes may be variably affected by insulin in different subjects. If hepatic or splanchnic triacylglycerol stores were used for VLDL synthesis, it would result in an observed lower level of newly made fatty acids secreted in VLDL. The intrahepatic lipogenic rate may still be up-regulated, but the rate of DNL fatty acids in the blood would appear lower if newly made fatty acids first joined a hepatic triacylglycerol pool that turned over slowly. A final critical factor that can affect a person’s lipogenic response to feeding is energy balance—a caloric deficit suppresses DNL, whereas overfeeding increases it (16). To control for this effect, the food intake of subjects was prescribed for the 3 d before the test to maintain energy balance. Furthermore, the amount of energy consumed in the liquid formula during the metabolic test was based on the subject’s energy need. Notably, the variability in the present study echoes that observed over a 24-h period by Hudgins et al (14) and remains an important topic for future research.

Although we cannot entirely explain the variability in postprandial DNL observed in our subjects, the results from the present study add substantial knowledge to the area of postprandial lipogenesis. The postprandial design of the study itself is relevant because most of the hours in the day are typically spent in the fed state. As cited above, one limitation of the present study design was the use of liquid meals rather than whole foods (7). However, the use of formula in the present study did provide a model for the stimulation of DNL when energy is provided in liquid form. Second, the frequency of meals in the present study may or may not be relevant when considering individual food intake patterns. The current results cannot discern between the effect on lipogenesis of bolus feeding (ie, 3 meals/d) compared with that of more frequent episodes of eating throughout the day, during which time insulin concentrations remain elevated over a longer duration.

In summary, the present work documents changes in rates of DNL postprandially in healthy men. The stimulation of lipogenesis after meals provided a significant source of fatty acids contributing to blood triacylglycerol, which averaged ≥23% VLDL-triacylglycerol fatty acids. Given the atherogenicity of the postprandial state, the contribution of DNL to this process requires further study. Identifying the individual factors that alter lipogenesis will be key to understanding how fatty acid synthesis fits into the syndrome of insulin resistance. The importance of DNL in elevating postprandial lipemia will best be delineated when it is quantitated along with the other sources of triacylglycerol in the blood after a meal (eg, NEFAs and dietary fatty acids). This will be the focus of future investigation.

We are grateful to the participants for contributing their time to the study. We thank the staff at Fairview University Medical Center, General Clinical Research Center, and Investigational Pharmacy, for their skilled clinical assistance. In particular, the contribution of Mary Coe was much appreciated. We also thank the numerous undergraduate students who provided significant laboratory assistance, Brian Barrows for subject recruitment, and Mary Gannon for insightful discussions of the data. EJP originally designed the study, provided data interpretation, and assisted with the writing of the manuscript. MTT coordinated the clinical research, data collection, technical and statistical analyses, and writing of the manuscript. The authors had no conflicts of interest.

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17. Marques-Lopes I, Ansorena D, Astiasaran I, Forga L, Martinez JA. Postprandial de novo lipogenesis and metabolic changes induced by a
Plasma antioxidant capacity in response to diets high in soy or animal protein with or without isoflavones

Sonia Vega-López, Kyung-Jin Yeum, Jaime L Lecker, Lynne M Ausman, Elizabeth J Johnson, Sridevi Devaraj, Ishwarlal Jialal, and Alice H Lichtenstein

ABSTRACT

Background: Several clinical trials have suggested that soy intake decreases oxidative stress. Soy isoflavones have antioxidant properties in vitro, but results of supplementation in clinical trials are inconclusive.

Objective: The objective was to evaluate the independent effects of soy protein and soy-derived isoflavones on plasma antioxidant capacity and biomarkers of oxidative stress.

Design: Forty-two hypercholesterolemic (LDL cholesterol > 3.36 mmol/L) subjects aged ≥50 y were provided with each of 4 diets in random order in a crossover design. Diets varied in protein source (10% of energy, soy or animal) and isoflavone content (trace or 50 mg/1000 kcal) and were consumed for 42 d each. Plasma antioxidants, protein carbonyls, malondialdehyde, total antioxidant performance, LDL oxidizability, and urinary F2-isoprostanes were measured at the end of each dietary phase.

Results: Plasma antioxidant concentrations were not significantly different, regardless of dietary treatment, except for isoflavones, which were higher after isoflavone supplementation ($P = 0.0001$). Although plasma total antioxidant performance was 10% higher with soy protein intake, regardless of dietary isoflavones ($P = 0.0003$), soy protein did not significantly affect most individual markers of oxidative stress (LDL oxidizability, urinary F2-isoprostanes, malondialdehyde, or protein carbonyls in native plasma). However, soy protein was associated with modestly lower concentrations of protein carbonyls in oxidized plasma. There was no significant effect of isoflavones on LDL oxidation, urinary F2-isoprostanes, or protein carbonyl groups, although, paradoxically, the plasma malondialdehyde concentration was significantly higher after the isoflavone-rich diets ($P = 0.04$).


KEY WORDS Animal protein, antioxidants, antioxidant capacity, cardiovascular disease, isoflavones, oxidative stress, soy protein

INTRODUCTION

Some epidemiologic data suggest that diets relatively high in soy protein are associated with a decreased relative risk of cardiovascular disease (CVD) and nonfatal myocardial infarction (1) and with lower total and LDL-cholesterol concentrations (2, 3). Early work has suggested a hypocholesterolemic effect of soy products (4), which in 1999 led to the authorization of a health claim for the cholesterol-lowering potential of soy products (5, 6). However, recent studies evaluating the lipid responses to soy consumption have only shown a modest (7–13) or no hypocholesterolemic (14–16) effect.

Several studies have suggested that soy intake aids against oxidative stress, as indicated on the basis of measurements of conjugated dienes in the LDL fraction (17, 18), lag time for copper-induced LDL oxidation (19–21), and plasma concentrations of F2-isoprostanes (19). However, results from other studies failed to support the antioxidant effects of soy (16, 22). The potential decrease in oxidative stress has been ascribed to the isoflavone component of the soybeans. Soybeans and products derived from soybeans represent the major source of dietary isoflavones (23). Compared with the major antioxidants in plasma (ascorbic acid, uric acid, α-tocopherol, β-carotene, and other carotenoids) (24, 25), the concentration of isoflavones in plasma is relatively low and can reach concentrations comparable with those of carotenoids [1 μmol/L (26), only after the consumption of meals containing soy products high in isoflavones or isoflavone supplements (8, 19, 26–30).

The 2 major isoflavones in soybeans are genistein and daidzein. Kerry and Abbey (31) reported that genistein inhibits copper- and perox radical–mediated LDL oxidation when added to a cell-free oxidation system but not when incorporated into LDL particles before the oxidation reaction. Kapiotis et al.

1 From the Cardiovascular Nutrition Laboratory (SV-L, JLL, LMA, and AHL) and the Carotenoids and Health Laboratory (K-JY and EJJ), Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University, Boston, and the Laboratory for Atherosclerosis and Metabolic Research, University of California, Davis Medical Center, Sacramento, CA (SD and IJ).

2 Based on work supported by the US Department of Agriculture under agreement no. 58-1950-4-401. Any opinions, findings, conclusion, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the US Department of Agriculture.

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43
Serum triacylglycerol (mmol/L) 1.54
Serum cholesterol (mmol/L)

**Study protocol**

Eighteen men and 24 postmenopausal women aged ≥50 y with LDL-cholesterol concentrations >3.36 mmol/L, but otherwise apparently healthy, were recruited from the greater Boston area. Participants were assigned to a sequence of four 42-d dietary periods: soy protein depleted of isoflavones (soy/−), soy protein enriched in isoflavones (soy/+), animal protein with no added isoflavones (animal/−), or animal protein with added isoflavones (animal/+ ) in a crossover design. All participants received each of the 4 diets in random order. The study protocol and diets were described in detail elsewhere (13). Baseline characteristics of the subjects are shown in Table 1. Written consent was obtained from all study volunteers. The study protocol was approved by the Human Investigation Review Committee of New England Medical Center and Tufts University.

All foods and beverages were provided to the study participants and caloric intakes were adjusted to maintain a stable body weight throughout the study. Diets were designed to have similar fatty acid profiles and contents of total fat, carbohydrate, protein, fiber, and cholesterol, which was confirmed by chemical analysis (Table 2). In diets containing isolated soy protein, 25 g/1000 kcal replaced common sources of animal protein from the diet. Diets containing isoflavones had 50 mg/1000 kcal, derived from the isolated soy protein preparation or added to the diet high in animal protein. The diet content of carotenoids, tocopherols, and retinol was calculated by using the Nutrition Data System for Research (NDS-R) software (version 4.04/32), which was developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis (36). A detailed description of the diets was previously reported (13).

Fasting (12 h) blood samples were collected on 3 separate days during the last week of each dietary phase. Plasma lipids were measured on all 3 d, and the mean was used for the statistical analysis. One 24-h urine collection was also obtained at the end of each dietary period. Plasma and urine samples were portioned and stored at −80 °C for subsequent analysis. Data on the effects of dietary protein type and isoflavones on the lipid profile were reported previously (13).

**Plasma antioxidants**

Plasma antioxidant concentrations were measured with an HPLC system as previously described, with minor modifications (26). Plasma samples (200 μL) were extracted with 2 mL chloroform:methanol (2:1, by vol) followed by 3 mL hexane. Samples were dried under nitrogen and resuspended in 150 μL ethanol:methyl-terbutyl ether (2:1, by vol), of which 50 μL was injected onto the HPLC. The HPLC system consisted of a Waters 600S Controller, a Waters 616 pump, a Waters 717 plus autosampler (Millipore, Milford, MA), a Waters 996 Photodiode Array Detector, a C30 carotenoid column (3 μm, 150 × 3.0 mm; YMC,}

### Table 1

<table>
<thead>
<tr>
<th>Baseline characteristics of the subjects</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>62.7 ± 8.8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.6 ± 3.4</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/L)</td>
<td>6.17 ± 0.78</td>
</tr>
<tr>
<td>Total</td>
<td>4.14 ± 0.65</td>
</tr>
<tr>
<td>HDL</td>
<td>1.32 ± 0.28</td>
</tr>
<tr>
<td>Serum triacylglycerol (mmol/L)</td>
<td>1.54 ± 0.66</td>
</tr>
</tbody>
</table>

*All values are x ± SD; n = 18 men and 24 postmenopausal women.*

### Table 2

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Soy/−</th>
<th>Soy/+</th>
<th>Animal/−</th>
<th>Animal/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate (% of energy)</td>
<td>45</td>
<td>46</td>
<td>47</td>
<td>48</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>17</td>
<td>16</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>Fat (% of energy)</td>
<td>38</td>
<td>37</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>Saturated</td>
<td>12.7</td>
<td>11.3</td>
<td>12.0</td>
<td>11.8</td>
</tr>
<tr>
<td>Monosaturated</td>
<td>14.7</td>
<td>14.4</td>
<td>14.2</td>
<td>14.3</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>6.1</td>
<td>6.1</td>
<td>5.6</td>
<td>5.7</td>
</tr>
<tr>
<td>Fiber (g/1000 kcal)</td>
<td>10</td>
<td>11</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Cholesterol (mg/1000 kcal)</td>
<td>151</td>
<td>150</td>
<td>154</td>
<td>168</td>
</tr>
<tr>
<td>α-Carotene (μg/1000 kcal)</td>
<td>431</td>
<td>431</td>
<td>279</td>
<td>279</td>
</tr>
<tr>
<td>β-Carotene (μg/1000 kcal)</td>
<td>1759</td>
<td>1759</td>
<td>826</td>
<td>826</td>
</tr>
<tr>
<td>Lycopene (μg/1000 kcal)</td>
<td>7490</td>
<td>7490</td>
<td>5577</td>
<td>5577</td>
</tr>
<tr>
<td>Lutein + zeaxanthin (μg/1000 kcal)</td>
<td>806</td>
<td>806</td>
<td>928</td>
<td>928</td>
</tr>
<tr>
<td>α-Tocopherol (mg/1000 kcal)</td>
<td>4.99</td>
<td>4.99</td>
<td>3.91</td>
<td>3.91</td>
</tr>
<tr>
<td>γ-Tocopherol (mg/1000 kcal)</td>
<td>5.40</td>
<td>5.40</td>
<td>4.73</td>
<td>4.73</td>
</tr>
<tr>
<td>Retinol (μg/1000 kcal)</td>
<td>172</td>
<td>172</td>
<td>202</td>
<td>202</td>
</tr>
<tr>
<td>Total isoflavones (mg/1000 kcal)</td>
<td>1.25</td>
<td>46.21</td>
<td>ND</td>
<td>51.71</td>
</tr>
<tr>
<td>Genistein (mg/1000 kcal)</td>
<td>0.37</td>
<td>27.17</td>
<td>ND</td>
<td>26.80</td>
</tr>
<tr>
<td>Daidzein (mg/1000 kcal)</td>
<td>0.84</td>
<td>13.89</td>
<td>ND</td>
<td>20.68</td>
</tr>
<tr>
<td>Glycitein (mg/1000 kcal)</td>
<td>ND</td>
<td>5.16</td>
<td>ND</td>
<td>4.23</td>
</tr>
</tbody>
</table>

*Macronutrients, fiber, cholesterol, and isoflavones were determined by chemical analysis of food; carotenoids, tocopherols, and retinol were calculated. Soy/−, soy protein depleted of isoflavones; soy/+, soy protein enriched in isoflavones; animal/−, animal protein with no added isoflavones; animal/+, animal protein with added isoflavones; ND, not detectable.*

One possible explanation for the discordant observations to date is that there is a potential independent effect of soy protein, soy-derived isoflavones, or the synergistic effect of both. This possibility has not been adequately addressed.

The aim of this study was to evaluate the effects of soy protein and soy-derived isoflavones, alone or in combination, on plasma antioxidant capacity and a wide range of biomarkers of oxidative stress in older adults with moderately elevated LDL-cholesterol concentrations.

**SUBJECTS AND METHODS**

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(32) also reported that genistein, but not daidzein, inhibits copper-mediated LDL oxidation, as shown by the inhibition of thiobarbituric acid–reactive substances and the formation of hydroperoxides in cell-free systems. In contrast with in vitro studies, data from interventional studies have failed to show an antioxidant effect from isoflavone supplements (33–35). One possibility for the discordant observations to date is that there is a potential independent effect of soy protein, soy-derived isoflavones, or the synergistic effect of both. This possibility has not been adequately addressed.

The aim of this study was to evaluate the effects of soy protein and soy-derived isoflavones, alone or in combination, on plasma antioxidant capacity and a wide range of biomarkers of oxidative stress in older adults with moderately elevated LDL-cholesterol concentrations.
SOY PROTEIN, ISOFLAVONES, AND ANTIOXIDANT CAPACITY

Analysis of soy/animal protein with added isoflavones.

Continuous absorbance monitoring at 234 nm as described earlier. The kinetics of conjugated dienes formation was assessed by isolation of LDL from plasma by sequential ultracentrifugation. The results are expressed as butyl hydroxytoluene equivalents.

Markers of oxidative stress

Plasma total antioxidant performance (TAP) was determined fluorometrically with a 1420-multilabel counter (Wallac Victor 2; Perkin-Elmer Life Sciences, Boston) as described by Aldini et al (25) with minor modifications. This method measures the rate of oxidation of 4,4-difluoro-5-(4-phenyl-1,3-butanediyl)-4-bora-3a,4a-diaza-s-indacene-3-undecenoic acid (BODIPY 581/591), a lipid-soluble fluorescent probe, and uses the lipid-soluble radical initiator 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN). Oxidation was determined by monitoring the appearance of green fluorescence of the oxidation product of BODIPY (λex = 500 nm, λem = 520 nm). The results are expressed as butyl hydroxytoluene equivalents.

LDL oxidizability was measured by monitoring the formation of conjugated dienes catalyzed by incubation with Cu2+ after the isolation of LDL from plasma by sequential ultracentrifugation. The kinetics of conjugated dienes formation was assessed by continuous absorbance monitoring at 234 nm as described earlier (38).

F2-Isoprostanes were measured in urine with the use of a previously described enzyme immunoassay (EIA) method (Cayman Chemicals, Ann Arbor, MI) (39). This EIA method was validated against a gas chromatography–mass spectrometry method (n = 68, R = 0.80) (39). F2-Isoprostanes were standardized by urinary creatinine measured by standard techniques.

Plasma protein carbonyls were measured with a modification of the enzyme-linked immunosorbent assay method as described by Marangon et al (40) before and after a 4-h incubation at 37 °C with 100 mmol/L of the aqueous free radical initiator 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH). Lipid peroxidation was assessed by measuring malondialdehyde with an HPLC system with a fluorescence detector, as previously described (41).

In vitro assessment of antioxidant capacity of purified isoflavones

The antioxidant capacity of the purified isoflavones added to the animal protein–based diet (Archer Daniels Midland Company, Decatur, IL) was tested in vitro with the TAP method described above. Plasma was incubated with isoflavones (0, 0.1, and 1 μmol/L) in the presence and absence of α-tocopherol (1 and 10 μmol/L).

Statistical analyses

Before the statistical analysis was conducted, variables with a skewed distribution (trans β-carotene, lutein, lycopene, and α-tocopherol) were log transformed to achieve normality. A repeated measures ANOVA was used to detect differences. Likewise, by design, plasma concentrations of trans β-carotene, lutein, lycopene, and α-tocopherol were log transformed to achieve normality before analysis. Soy/−, soy protein depleted of isoflavones; soy/+ , soy protein enriched in isoflavones; animal/− , animal protein with no added isoflavones; animal/+ , animal protein with added isoflavones.

RESULTS

Plasma antioxidants

As by design, plasma genistein and daidzein were significantly higher during the isoflavone-supplemented periods (Table 3), which reflects compliance with the intervention regimen. Likewise, by design, plasma concentrations of trans β-carotene, lutein, lycopene, retinol, α-tocopherol, and γ-tocopherol were relatively high and, although statistically significant differences were identified between the study phases, the magnitude of the

### Table 3

<table>
<thead>
<tr>
<th>Variable</th>
<th>Soy/−</th>
<th>Soy/+</th>
<th>Animal/−</th>
<th>Animal/+</th>
<th>Protein</th>
<th>Isoflavones</th>
<th>Protein × isoflavones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein (nmol/L)</td>
<td>50 ± 37</td>
<td>617 ± 498</td>
<td>7 ± 12</td>
<td>695 ± 443</td>
<td>0.5269</td>
<td>0.0001</td>
<td>0.2736</td>
</tr>
<tr>
<td>Daidzein (nmol/L)</td>
<td>15 ± 9</td>
<td>139 ± 91</td>
<td>3 ± 5</td>
<td>275 ± 211</td>
<td>0.0050</td>
<td>0.0001</td>
<td>0.0030</td>
</tr>
<tr>
<td>trans β-Carotene (μmol/L)</td>
<td>1.04 ± 0.89</td>
<td>1.12 ± 0.77</td>
<td>1.19 ± 1.02</td>
<td>1.13 ± 1.03</td>
<td>0.6910</td>
<td>0.9529</td>
<td>0.0130</td>
</tr>
<tr>
<td>Lutein (μmol/L)</td>
<td>0.30 ± 0.14</td>
<td>0.24 ± 0.11</td>
<td>0.29 ± 0.16</td>
<td>0.30 ± 0.17</td>
<td>0.1157</td>
<td>0.0004</td>
<td>0.0118</td>
</tr>
<tr>
<td>Lycopene (μmol/L)</td>
<td>1.60 ± 0.74</td>
<td>1.45 ± 0.66</td>
<td>1.62 ± 0.71</td>
<td>1.50 ± 0.61</td>
<td>0.4150</td>
<td>0.0475</td>
<td>0.8904</td>
</tr>
<tr>
<td>Retinol (μmol/L)</td>
<td>2.17 ± 0.53</td>
<td>2.19 ± 0.51</td>
<td>2.20 ± 0.46</td>
<td>2.21 ± 0.59</td>
<td>0.6074</td>
<td>0.8843</td>
<td>0.9502</td>
</tr>
<tr>
<td>α-Tocopherol (μmol/L)</td>
<td>35.5 ± 10.3</td>
<td>34.3 ± 11.2</td>
<td>36.7 ± 10.3</td>
<td>35.5 ± 12.8</td>
<td>0.1308</td>
<td>0.1329</td>
<td>0.8965</td>
</tr>
<tr>
<td>γ-Tocopherol (μmol/L)</td>
<td>5.30 ± 2.08</td>
<td>4.91 ± 1.76</td>
<td>5.06 ± 2.14</td>
<td>4.89 ± 1.41</td>
<td>0.5012</td>
<td>0.1926</td>
<td>0.4645</td>
</tr>
</tbody>
</table>

1 n = 42. Variables with a skewed distribution (trans β-carotene, lutein, lycopene, and α-tocopherol) were log transformed to achieve normality before analysis. Soy/−, soy protein depleted of isoflavones; soy/+ , soy protein enriched in isoflavones; animal/− , animal protein with no added isoflavones; animal/+ , animal protein with added isoflavones.

2 A repeated-measures ANOVA was used to detect differences.

3 ± SD (all such values).
differences would not be predicted to have a physiologically significant effect. Standardization of plasma antioxidants on the basis of total cholesterol concentrations did not significantly alter the results (data not shown).

**Plasma total antioxidant performance**

As shown in Figure 1, plasma TAP was modestly higher (10%) at the end of the soy-protein phases than during the animal protein phases, regardless of the isoflavone content of the diet.

**Biomarkers of oxidative stress**

The different biomarkers of oxidative stress at the end of each dietary phase are depicted in Table 4. There were no significant effects of dietary protein or isoflavones on the susceptibility of LDL to oxidation measured by detection of conjugated diene formation (lag time and oxidation rate) or on in vivo lipid peroxidation, assessed by measuring urinary F₂-isoprostanes. There was no significant effect of protein source or isoflavone intake on native protein carbonyl groups. However, animal protein intake was associated with a significantly higher concentration of protein carbonyl groups after incubation of plasma with the radical initiator AAPH. Plasma malondialdehyde concentration, a marker of lipid peroxidation, was 10% higher after the isoflavone-supplemented periods.

**In vitro assessment of TAP of isolated isoflavones**

Incubation of plasma with isoflavones before the TAP analysis at concentrations of 0.1, 0.5, 1.0, and 10 μmol/L resulted in an increase in TAP of 7%, 8%, 10%, and 11%, respectively (P > 0.05 for all). Incubation of plasma with α-tocopherol at 1.0 and 10 μmol/L resulted in an increase in TAP of 10% and 17%, respectively (P > 0.05 for both). Further addition of isoflavones (0.1 and 1 μmol/L) to the 1 μmol/L α-tocopherol system provided an additional increase in TAP of 14% (P = 0.03) and 24% (P = 0.01), respectively. In the presence of 10 μmol α-tocopherol/L, TAP was further increased by 21% (P = 0.0002) and 26% (P = 0.001) when coincubated with 0.1 and 1 μmol isoflavones/L, respectively.

**DISCUSSION**

This study assessed the independent and combined effects of soy protein and soy-derived isoflavones on antioxidant capacity and biomarkers of oxidative stress in adults with moderately elevated LDL-cholesterol concentrations. The current data suggest a modest difference in plasma TAP between the 2 types of protein investigated, independent of isoflavones intake. Similar to the current data, a multiple regression analysis showed that after 12 wk of treatment, plasma total antioxidant status was positively affected by soy protein but not by soy isoflavones (22). These results are supported by reports suggesting that soy-protein isolate and a soy peptide were effective in reducing paraquat-induced oxidative stress in rats (42, 43). The antioxidant activity of soy protein has also been attributed to the iron-chelating properties of its phytic acid. Previously, Swain et al (22) reported that supplementation with 40 g protein/d provided as isoflavone-rich soy-protein isolate, isoflavone-poor soy protein isolate, or whey protein did not significantly affect plasma total antioxidant status in a double-blind parallel trial. The design of the current study did not allow the determination of which protein type induced the differences observed.

Plasma antioxidants other than isoflavones also contribute to the total antioxidant capacity and, in absolute concentration,
actually exceed that of the isoflavones. In the current study, all diets were formulated to be rich in fruit and vegetables and, therefore, relatively rich in antioxidants. Despite the somewhat higher content of lycopene, α-carotene, β-carotene, α-tocopherol, and γ-tocopherol in the soy protein–containing diets, differences observed in plasma trans β-carotene, lutein, and lycopene concentrations were small and not significantly associated with type of protein. Lycopene and trans β-carotene concentrations were above the 90th percentile reported for subjects aged 50–70 y in the third National Health and Nutrition Examination Survey (NHANES III) during all dietary periods. Similarly, plasma tocopherol concentrations were above the 75th percentile throughout the study.

In the current study, soy-protein intake was only associated with a lower concentration of protein carbonyl groups after incubation of plasma with a water-soluble radical initiator (AAPH), compared with animal protein, whereas no other effects on biomarkers of oxidative stress were observed. This observation extends the results reported by Steinberg et al (16). After the provision of soy or milk supplements, with and without isoflavones, no significant effect on copper-mediated LDL susceptibility to oxidation was observed. In contrast, Ashton et al (21) reported a longer lag phase of copper-induced LDL oxidation after meat was substituted with tofu. In a small study, Tikkanen et al (20) documented that the consumption of 3 soy bars per day (21 g protein, 36 mg genistein, and 21 mg daidzein) for 2 wk resulted in a longer lag phase of copper-induced LDL oxidation than that at baseline. Jenkins et al (17, 18) reported a decrease in LDL conjugated dienes after consumption of a minimum of 36 g soy protein/d, regardless of the isoflavone content of the foods. Of these reports, only the report of Steinberg et al (16) included information on dietary antioxidant intake and reported a high intake of dietary antioxidants, similar to that in the current study. It is possible that the lack of effect of soy consumption on biomarkers of oxidative stress in both studies was masked by the relatively high concentration of other plasma antioxidants. Discrepancies in the effects observed in other studies are hard to interpret because of the use of a wide variety of soy products providing from 15 to 52 g soy protein/d, each containing different amounts of isoflavones.

Although in vitro measures have suggested an antioxidant effect of isolated isoflavones (31, 32), the in vivo evidence is equivocal. No differences in urinary F₂-isoprostanes or LDL oxidation (lag time or oxidation rate) were observed in the current study. In contrast, high (56 mg) compared with low (2 mg) daily isoflavone intakes resulted in lower concentrations of plasma 8-epi-prostaglandin F₂α, a biomarker of in vivo lipid peroxidation, and longer lag times for LDL oxidation (19). However, other studies have failed to find an antioxidant effect of soy-derived isoflavones when provided as supplements, using either ex vivo LDL oxidizability (33, 34) or urinary F₂-isoprostanes (35) as biomarkers of oxidative stress.

In the current study, malondialdehyde concentrations—an indirect marker of lipid peroxidation—were unexpectedly higher after the isoflavone-supplemented dietary periods. Wiseman et al (19) reported that plasma malondialdehyde did not significantly differ regardless of dietary isoflavones. However, in their study, the malondialdehyde concentration was 6% higher after the high-isoflavone period, albeit not significant. Despite the similar amounts of dietary isoflavones between the 2 studies, participants in the current study were older (mean age of 63 y compared with 30 y), had slightly lower plasma isoflavone concentrations, and had higher plasma malondialdehyde concentrations (almost 4 times those in Wiseman et al’s study), regardless of dietary period. Perhaps more importantly, plasma cholesterol concentrations were higher in the current study, which likely contributed to increased oxidative stress.

In contrast with the human data, in vitro and animal studies have more consistently reported an antioxidant effect of soy-derived isoflavones. Kerry and Abbey (31) reported that the addition of genistein to human LDL resulted in lower malondialdehyde concentrations over an 8-h azo-initiated oxidation incubation. Similarly, animal studies have shown lower malondialdehyde concentrations in plasma (44) and in the aortic arch (45) of animals fed high doses of isoflavones. Noteworthy, the concentrations of isoflavones used in both in vitro and animal studies are higher than what can be achieved in the plasma of subjects consuming diets supplemented with isoflavones, as reported in the current study and in similar studies (19). Although the higher malondialdehyde concentrations observed after the subjects consumed the isoflavone-containing diets in the current study is of interest, it could not be determined whether isoflavone intake led to a greater extent of lipid peroxidation or was secondary to other reactions (46).

The lack of a protective effect of isoflavones against LDL oxidizability might be related to their relative hydrophobicity (47). Meng et al (48) reported that in vitro free genistein and daidzein are only incorporated into LDL to a small extent and do not significantly influence oxidation resistance measured by copper-induced conjugated diene formation and that they require esterification to become more readily incorporated into LDL. Therefore, because of the limited incorporation of isoflavones into LDL, any measures that involve the isolated lipoprotein need to be interpreted cautiously. In addition, because some of the isoflavones in plasma could be in the conjugated form, they may not have been available to function as an antioxidant (49).

This study had several limitations. The possibility cannot be ruled out that the antioxidant effect of α-tocopherol and other antioxidants present in high concentrations in plasma may have masked any potential effects of isoflavones. This possibility is supported by the results from the in vitro assessment of the antioxidant capacity of isoflavones, which suggest that the small additive antioxidant effect of isoflavones is greater when α-tocopherol concentrations are lower (1 μmol/L). Moreover, the possible interaction between different antioxidants in vivo may enhance the overall antioxidant status, making the evaluation of the effect of particular antioxidants hard to interpret.

In conclusion, consumption of diets rich in soy protein and soy-derived isoflavones do not appear to decrease oxidative stress. Despite the modest increase in plasma TAP associated with soy-protein intake, this finding was not reflected in specific effects on measures of oxidative stress as potentially affected by either soy protein or soy-derived isoflavones. The presence of other antioxidants from a nutritionally adequate diet may have mitigated the antioxidant effect of isoflavones previously reported in in vitro systems.

We thank Susan Jalbert for her outstanding technical assistance, Majella O’Keefe for her assistance with the plasma antioxidant measurements, Herman Adlercreutz for the measurement of plasma isoflavones, and Paul Jacques for making the NHANES III data available. The cooperation of the study subjects was gratefully acknowledged.
REFERENCES


n–3 Fatty acids consumed from fish and risk of atrial fibrillation or flutter: the Danish Diet, Cancer, and Health Study\textsuperscript{1–3}

\textbf{Lars Frost and Peter Vestergaard}

\textbf{ABSTRACT}

\textbf{Background:} Experimental studies have shown that n–3 polyunsaturated fatty acids in fish may have antiarrhythmic properties.

\textbf{Objective:} We examined the association between consumption of n–3 fatty acids from fish and risk of atrial fibrillation or flutter.

\textbf{Design:} In a prospective cohort study of 47,949 participants (mean age: 56 y) in the Danish Diet, Cancer, and Health Study, we investigated the relation between the consumption of n–3 fatty acids from fish estimated from a detailed semiquantitative food questionnaire and risk of atrial fibrillation or flutter. The subjects were followed up in the Danish National Registry of Patients for the occurrence of atrial fibrillation or flutter and in the Danish Civil Registration System (vital status and emigration). The consumption of n–3 fatty acids from fish was analyzed as sex-specific quintiles with the use of Cox proportional hazards models.

\textbf{Results:} During follow-up (\(\bar{x}\): 5.7 y), atrial fibrillation or flutter had developed in 556 subjects (374 men and 182 women). When the lowest quintile of n–3 fatty acids consumed from fish was used as a reference, the unadjusted hazard rate ratios in quintiles 2, 3, 4, and 5 were 0.93, 1.11, 1.10, and 1.44, respectively (\(P\) for trend = 0.001). The corresponding adjusted hazard rate ratios were 0.86, 1.08, 1.01, and 1.34 (\(P\) for trend = 0.006). Inclusion of information on the frequency of fatty fish consumption did not alter these associations.

\textbf{Conclusions:} Consumption of n–3 fatty acids from fish was not associated with a reduction in risk of atrial fibrillation or flutter. We cannot exclude the possibility of residual confounding caused by a lack of information on intake of fish-oil tablets. \textit{Am J Clin Nutr} 2005;81:50–4.

\textbf{KEY WORDS} Arrhythmia, cohort studies, diet, epidemiology, fish oils, nutrition, Danish Diet, Cancer, and Health Study

\textbf{INTRODUCTION}

Experimental studies have suggested that n–3 polyunsaturated fatty acids, mainly from fish or fish-oil capsules, may have antiarrhythmic properties (1–5). Recently, it was confirmed that n–3 fatty acids block the cardiac Na\textsuperscript{+} channels in rat ventricular myocytes (6), and a small nonblinded, nonrandomized pilot study in 10 patients with an implanted cardioverter defibrillator showed a reduction in the inducibility of sustained ventricular tachycardia after infusion of n–3 fatty acids (7). We therefore examined the association between consumption of n–3 polyunsaturated fatty acids from fish and the risk of atrial fibrillation or flutter in the Danish Diet, Cancer, and Health Study.

\textbf{SUBJECTS AND METHODS}

\textbf{Study population}

The Danish Diet, Cancer, and Health Study is a prospective cohort study with the primary aim of studying the role of diet in cancer risk but with a potential for studying other diseases as well. The study design was described in detail elsewhere (8, 9).

From December 1993 through May 1997, 80,996 men and 79,729 women aged 50 to 64 y were invited to participate in the study; 27,177 men and 29,876 women accepted the invitation. Eligible cohort members were born in Denmark, living in the Copenhagen and Aarhus areas, and had no previous cancer diagnosis in the Danish Cancer Registry. The baseline data were linked to the Danish Cancer Registry and other population-based registries, including the Danish National Registry of Patients, and the Danish Civil Registration System, using the civil registry number, which is a unique number given to everyone with an address living in Denmark since 1968. The Civil Registration System has electronic records of all changes in vital status for the Danish population since 1968, including change in address, date of emigration, and date of death. The Danish National Registry of Patients was established in 1977, and has records for 99.4% of all discharges from nonpsychiatric hospitals in Denmark (10). The data include the civil registry number, dates of admission and discharge, surgical procedures performed, and up to 20 discharge diagnoses per discharge, classified until 1993 according to the Danish version of the International Classification of Diseases, 8th revision, and thereafter according to the national version of International Classification of Diseases, 10th revision. The physician who discharged the patient coded all discharge diagnoses.

To study incident cases of atrial fibrillation, and to reduce confounding, we excluded participants who had been hospitalized before baseline with endocrine diseases or cardiovascular diseases others than hypertension, recorded in the National Registry of Patients, International Classification of Diseases, 8th revision codes: 240–279, 390–398, and 410–458, and International...
Classification of Diseases, 10th revision codes: E00-E90, I00-I09, I16-I99. We did not exclude patients with hypertension before or at baseline for several reasons. First, the diagnostic criteria for hypertension have changed over the last decades. Second, the validity of a diagnosis of hypertension in the Danish National Registry of Patients is low (11). Third, if we used a definition of hypertension as a systolic blood pressure $>140$ mm Hg at baseline, we would exclude $>50\%$ of subjects from the cohort (8). Finally, we did not a priori feel that the relation between blood pressure and risk of atrial fibrillation included a threshold function, which allowed us to exclude any subject from the cohort who had a specific level of blood pressure. The Danish Diet, Cancer, and Health Study and the present study were approved by the Regional Ethics Committees in Copenhagen and Aarhus and by The Danish Data Protection Agency.

Baseline data

Height, weight, systolic and diastolic blood pressure, and total serum cholesterol were measured at baseline. Body weight was measured with the use of a digital scale weight (Soehnle, Germany) and was recorded to the nearest 100 g. Blood pressure (systolic and diastolic) were measured with an automatic blood pressure measurement device (Takeda UH 751, Tokyo). Non-fasting total serum cholesterol was measured according to national guidelines (12).

All participants filled in a questionnaire about medical diseases, including myocardial infarction, angina, stroke, hypertension, hypercholesterolemia, and diabetes, and drug treatment for those conditions. Subjects who reported to have ischemic heart disease, stroke, or diabetes who were medicated for those conditions were excluded from the present study. The participants also completed a questionnaire about smoking habits, alcohol intake, health, and duration of education.

Dietary intake of $n-3$ fatty acids from fish

All cohort members completed a detailed semiquantitative food- and drink-frequency questionnaire. The study participants were asked to fill in a questionnaire about type and frequency of fish consumption (never, less than once per month, once per month, 2–3 times/mo, once per week, 2–4 times/wk, 5–6 times/wk, once per day, 2–3 times/d, 4–5 times/d, 6–7 times/d, and ≥8 times/d). The daily intake of specific foods and nutrients was computed from the food-frequency questionnaire for each participant with the use of the software program FOODCALC (13). Standard recipes and sex-specific portion sizes were applied to calculate intake in grams per day with the use of data from different sources, ie, the 1995 Danish National Dietary Survey (14), 24-h diet-recall interviews from 3818 of the study participants (15), and various cookery books.

Descriptions of the development and validation of the food-frequency questionnaire were published previously (16, 17). A biomarker study was carried out to validate the information on dietary intake of fatty acids, including marine $n-3$ polyunsaturated fatty acids. This study showed correlation coefficients (Pearson) between the reported dietary intake in the food-frequency questionnaire and the relative fat tissue composition of the marine fatty acids eicosapentaenoic acid and docosahexaenoic acid of 0.47 and 0.41, respectively (18). The exposure evaluated in the present study was the consumption of marine $n-3$ fatty acids from fish estimated from the food-frequency questionnaire.

We categorized the following species of fish (which are available and eaten in Denmark) as fatty fish: herring, mackerel, sardine, trout, and salmon. We converted the exposure information into 2 variables: 1) the amount (g) of marine $n-3$ polyunsaturated fatty acids consumed (per day) from any species of fish and evaluated as sex-specific quintiles and 2) the frequency of consumption of fatty fish (<2 or ≥2 times/wk).

Identification of incident atrial fibrillation and atrial flutter

The general health and hospital care systems in Denmark are no-charge and nonprofit systems that are financed through taxes. During the study period there was a very limited capacity in private specialist practices and in private hospitals. We identified probable cases of atrial fibrillation or flutter within the cohort in The Danish National Registry of Patients, ie, cases with the discharge diagnoses 427.93, 427.94, and 148 through 31 December 2001. Beginning from 1 January 1995, patients who were only seen in an outpatient hospital clinic were also coded into the Danish National Registry of Patients. A change from the International Classification of Diseases 8th revision to the International Classification of Diseases 10th revision occurred in Denmark in 1994. Atrial fibrillation and atrial flutter were coded separately in the International Classification of Diseases 8th revision (codes 427.93 and 427.94); however, in the International Classification of Diseases 10th revision, atrial fibrillation and flutter have the same code (I48).

A single reviewer (LF), using a standardized form, reviewed the medical records of the subset of study participants living in the county of Aarhus with an incident diagnosis of atrial fibrillation or flutter recorded in the Danish National Registry of Patients through December 1999. Of 116 subjects with an incident diagnosis of atrial fibrillation or flutter, an electrocardiogram, a printout from telemetry, or a printout from a Holter recording verified atrial fibrillation or atrial flutter in 112 subjects. Thus, diagnoses for 112 of the 116 subjects (97%) were verified. Of the 112 subjects with a verified diagnosis, 103 subjects (92%) had atrial fibrillation, 3 (3%) had both atrial fibrillation and atrial flutter, and 6 (5%) had atrial flutter.

Follow-up

The study participants were followed-up in the National Registry of Patients and in the Central Person Registry. Linking was done by using the civil registry number. Follow-up for atrial fibrillation or flutter began on the date of visit to one of the study centers and ended on the date of an event or a censoring (ie, a diagnosis of atrial fibrillation or flutter, death, emigration, or 31 December 2001, whichever came first).

Statistical methods

We used piecewise linear regression to examine the relation between a continuous variable and the hazard of atrial fibrillation or flutter (19). We kept a continuous variable as continuous in the Cox regression model, when appropriate, according to these analyses.

We computed a multivariate Cox regression model by an initial forced entry of known risk factors for atrial fibrillation, namely age, sex, body height, body mass index, alcohol consumption, systolic blood pressure, and treatment for hypertension, followed by forward selection of other variables of interest.
TABLE 1
Baseline characteristics of the subjects in the Danish Diet, Cancer, and Health Study according to sex-specific quintiles of n−3 polyunsaturated fatty acid (PUFA) intake from fish

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Quintile 1 (n = 9589)</th>
<th>Quintile 2 (n = 9590)</th>
<th>Quintile 3 (n = 9591)</th>
<th>Quintile 4 (n = 9590)</th>
<th>Quintile 5 (n = 9589)</th>
<th>P for correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>n−3 PUFA intake from fish (g/d)</td>
<td>0.16 ± 0.08</td>
<td>0.36 ± 0.06</td>
<td>0.52 ± 0.07</td>
<td>0.74 ± 0.10</td>
<td>1.29 ± 0.47</td>
<td>—</td>
</tr>
<tr>
<td>Intake of fatty fish ≥ 2 times/wk (%)</td>
<td>0.4</td>
<td>12.2</td>
<td>50.4</td>
<td>84.1</td>
<td>96.6</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Total energy intake (kJ/d)</td>
<td>8748 ± 2531</td>
<td>9127 ± 2438</td>
<td>9559 ± 2496</td>
<td>10053 ± 2576</td>
<td>11140 ± 3045</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Age (y)</td>
<td>55.5 ± 4</td>
<td>55.7 ± 4</td>
<td>55.9 ± 4</td>
<td>56.2 ± 4</td>
<td>56.9 ± 4</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Sex, men (%)</td>
<td>47.0</td>
<td>47.0</td>
<td>47.0</td>
<td>47.0</td>
<td>47.0</td>
<td></td>
</tr>
<tr>
<td>Body height (cm)</td>
<td>169.9 ± 9</td>
<td>170.2 ± 9</td>
<td>170.2 ± 9</td>
<td>170.2 ± 9</td>
<td>170.0 ± 9</td>
<td>0.35</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.9 ± 4.0</td>
<td>25.9 ± 4.0</td>
<td>25.9 ± 3.9</td>
<td>25.9 ± 3.9</td>
<td>25.9 ± 4.0</td>
<td>0.08</td>
</tr>
<tr>
<td>Smoking²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never (%)</td>
<td>37</td>
<td>36</td>
<td>38</td>
<td>36</td>
<td>35</td>
<td>—</td>
</tr>
<tr>
<td>Former (%)</td>
<td>27</td>
<td>28</td>
<td>29</td>
<td>29</td>
<td>28</td>
<td>—</td>
</tr>
<tr>
<td>Current (%)</td>
<td>36</td>
<td>35</td>
<td>34</td>
<td>35</td>
<td>37</td>
<td>0.12</td>
</tr>
<tr>
<td>Alcohol intake (g/d)</td>
<td>18.4 ± 22</td>
<td>20.2 ± 21</td>
<td>21.0 ± 21</td>
<td>21.6 ± 21</td>
<td>21.8 ± 22</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>138 ± 20</td>
<td>139 ± 20</td>
<td>139 ± 20</td>
<td>139 ± 20</td>
<td>140 ± 20</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Treatment for hypertension (%)</td>
<td>9.9</td>
<td>9.8</td>
<td>10.0</td>
<td>10.4</td>
<td>11.4</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Total cholesterol &gt; 6 mmol/L (%)</td>
<td>48.1</td>
<td>49.0</td>
<td>48.7</td>
<td>50.4</td>
<td>52.4</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

1 Spearman correlation analysis. Not all significant digits are shown in the table (ie, values have been rounded). Because of the large number of subjects, some of the significant differences were due to decimal places not shown.
2 ± SD (all such values).
3 Sum of percentages may not add up to 100 because of rounding.

We performed supplementary analyses by adding product terms to test for interaction. The relevance of a variable in the model was further assessed by the change-in-estimate method (20). The variables included in our final model were age (y), sex, body height (cm), body mass index (kg/m²), systolic blood pressure (mm Hg), treatment for hypertension (yes or no), total serum cholesterol > 6 mmol/L (yes or no), alcohol consumption (g/d), and sex-specific quintile of fish-oil consumption. Thereafter, we assessed further potential confounders: total daily energy intake (kJ/d), frequency of consumption of fatty fish, smoking (never, former, or current), and length of education after elementary school (0, <3, 3–4, or ≥4 y) to evaluate the potentials for a change in the estimate of the hazards for atrial fibrillation or flutter associated with consumption of fish oil.

Correlation was evaluated by Spearman’s nonparametric correlation analysis. Tests for linear trend were calculated by assigning the medians of intake in quintiles treated as a continuous variable. The assumption of proportional hazards in the Cox models was evaluated with the use of graphic assessment and was found to be appropriate in all models. We calculated 95% CIs throughout the analyses. We used SPSS statistical software version 11.5 (SPSS Inc, Chicago).

RESULTS
The cohort included 57 053 subjects at baseline. We excluded 9022 subjects who reported to have taken or who were taking medicine for endocrine or cardiovascular diseases (hypertension was not excluded), to have a diagnosis of endocrine or cardiovascular disease (hypertension was not excluded) in the Danish National Registry of Patients before or at baseline, or who met both criteria. Eighty-two subjects were excluded because of missing information on fish-oil consumption. Thus, the study population included in the cohort consisted of 47 949 subjects, 22 528 men and 25 421 women. The men provided a total of 128 131 person-years of risk (x: 5.7 y; range: 0–8.1 y), and the women contributed 147 251 person-years of risk (x: 5.7 y; range: 0–8.1 y). During follow-up, 374 men (1.7%) and 182 women (0.7%) had an incident diagnosis of atrial fibrillation or flutter in the National Registry of Patients, which corresponded with incidence rates of 29.1/10 000 person-years at risk in men and 12.4/10 000 person-years at risk in women.

Characteristics at baseline according to sex-specific quintiles of consumption of n−3 fatty acids from fish are shown in Table 1. The mean consumption of fish oil was 0.16 g/d in the lowest quintile compared with 1.29 g/d in the top quintile. Mean age, consumption of alcohol, systolic blood pressure, and the proportion of subjects receiving treatment for hypertension increased by increasing category of fish-oil consumption.

The incidence rates by increasing sex-specific quintiles of fish-oil consumption were 18.1, 16.7, 20.0, 20.0, and 26.2 per 10 000 person-years of follow-up (Table 2). The risk of atrial fibrillation or flutter according to sex-specific quintiles of intake of marine n−3 polyunsaturated fatty acids is shown in Table 2. When the lowest sex-specific quintile of fish-oil consumption was used as a reference, the adjusted hazard rate ratios in quintiles 2, 3, 4, and 5 were 0.86, 1.08, 1.01, and 1.34 (P value for trend 0.006). Information on the frequency of consumption of fatty fish (≥2 times/wk or <2 times/wk) did not change these estimates.

The risk of atrial fibrillation or flutter associated with exposure to n−3 fish oil from consumed fish did not change substantially by exposure to specific types of fish (mackerel, salmon, or herring) or by specific method of food preparation (data not shown). We did not observe any effect modification by sex or any other confounding variables.

DISCUSSION
In this large cohort study, we found that consumption of n−3 fatty acids from fish did not reduce the risk of atrial fibrillation or flutter. The lack of effect of fish oil in our study could have been...
because the consumption of fish oil was insufficient to prevent arrhythmias. However, the mean consumption of marine n-3 polyunsaturated fatty acids in the top quintile was >1 g/d in men as well as in women, which was comparable with the daily supplement of 1 g n-3 polyunsaturated fatty acids used in the GISSI-Prevenzione Trial (2). Because we excluded subjects with known heart disease from our cohort, we cannot exclude the possibilities that fish oil may prevent the development of atrial fibrillation in patients with symptomatic heart disease or that fish oil may prevent relapses of atrial fibrillation in patients with paroxysmal atrial fibrillation.

The main strengths of our study were the large number of cases with atrial fibrillation; the detailed information on potential confounding factors, the complete follow-up through nationwide; population-based registries, which limit selection and surveillance bias; the standardized assessment of a sample of the registered outcome events; and a uniformly organized no-charge and nonprofit health care system. We used restriction in applying admissibility criteria to reduce confounding and to increase validity (21).

We had limited statistical power, and we were only able to include atrial fibrillation or flutter that was symptomatic and led to hospitalization or clinical investigation in an outpatient hospital clinic. However, given the age profile of our study cohort, it is likely that patients with symptoms of atrial fibrillation were referred to a hospital for further evaluation, and, during the study period, there was a limited capacity in private clinics and hospitals. We relied on self-reported data on consumption of fish, and we had no information on supplementary intake of fish oil from fish-oil capsules. However, a recent study on characteristics associated with the use of dietary supplements concluded that taking fish-oil supplements was associated with eating oil-rich fish (22). Thus, the problem in the present study does not seem to be misclassification of low exposure as high exposure and vice versa, because those already exposed to n-3 fish oil from eating fish may have added an additional exposure by taking fish-oil tablets. We recognize that some subjects categorized in the lowest quintiles of fish-oil intake may have taken fish-oil tablets, and this causes misclassification of exposure, which introduces bias toward unity. However, the major question raised in our study was as follows: do n-3 fatty acids from fish protect against atrial fibrillation? If fish oil has a protective role, we would have expected it to be seen in the top quintile of exposure, especially because we know that the consumption of fish-oil tablets is highest among those who eat much fatty fish.

Because of the multiple (≥2) levels of exposure, bias introduced by misclassification of exposure may be toward as well as away from the null, depending on the categories to which the individuals are misclassified (21). Biased follow-up may have occurred if the unexposed subjects were underdiagnosed for atrial fibrillation or flutter more than were the exposed subjects (21). In the unadjusted analyses we found that increasing consumption of fish oil was associated with increased risk of atrial fibrillation or flutter. We also found that increasing intake of fish oil was associated with a slightly older age and with more subjects receiving treatment for hypertension. Older age and hypertension are not only risk factors for atrial fibrillation but are also risk factors for hospitalization, which often leads to the recording of an electrocardiogram, which will increase the probability of the detection of asymptomatic atrial fibrillation or flutter.

Substantial geographic variation exists in total fish intake and intake of fatty fish between different countries. Of the 10 European countries participating in the European Investigations into Cancer and Nutrition (EPIC) Study, the greatest intake of very fatty fish was in the coastal areas of northern Europe (Denmark, Sweden, and Norway) (23). The intake of fish oil in Denmark is also higher than the mean intake of fish oil in the United States (4). We do not know whether fish oil has a protective effect against the occurrence of atrial fibrillation in populations with a low intake of fatty fish.

We thank Katja Boll (programmer) and Jytte Fogh Larsen (secretary) from The Danish Cancer Society for assisting with the data collection, Anne Tjønneland and Kim Overvad for access to data, and Lone Juul Hune (UNI-C, the Danish Information Technology Centre for Education and Research) for assisting with the data management and statistical analyses.

LF designed the study, analyzed the data, and prepared the manuscript. PV reviewed the data analyses and the manuscript. LF received funding or grant support for research projects from Boehringer-Ingelheim, Cardiome Pharma, The Danish Heart Foundation, The Danish Medical Research Council, The Danish Society of Nephrology, The Hørsholm Foundation, Laerdal’s Foundation for Acute Medicine, Løgkedersforeningens Forskningsfond, Merk Sharp & Dome, Nycomed, and Pfizer, and he serves or has served as a consultant for AstraZeneca and Pfizer. PV had no conflict of interest.

### Table 2

<table>
<thead>
<tr>
<th>n-3 PUFA intake from fish (g/d)</th>
<th>Quintile 1 (n = 9589)</th>
<th>Quintile 2 (n = 9590)</th>
<th>Quintile 3 (n = 9591)</th>
<th>Quintile 4 (n = 9590)</th>
<th>Quintile 5 (n = 9589)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean duration of follow-up (y)</td>
<td>5.8 ± 0.76</td>
<td>5.7 ± 0.73</td>
<td>5.7 ± 0.73</td>
<td>5.7 ± 0.73</td>
<td>5.7 ± 0.73</td>
</tr>
<tr>
<td>Person-years of follow-up (y)</td>
<td>55 193</td>
<td>55 013</td>
<td>54 766</td>
<td>55 111</td>
<td>55 051</td>
</tr>
<tr>
<td>Subjects with atrial fibrillation or flutter (n)</td>
<td>100</td>
<td>92</td>
<td>110</td>
<td>110</td>
<td>144</td>
</tr>
<tr>
<td>Incidence rate per 10 000 person-years</td>
<td>18.1</td>
<td>16.7</td>
<td>20.0</td>
<td>20.0</td>
<td>26.2</td>
</tr>
<tr>
<td>Unadjusted hazard rate ratio (95% CI)</td>
<td>1.00</td>
<td>0.93 (0.70, 1.23)</td>
<td>1.11 (0.85, 1.46)</td>
<td>1.10 (0.84, 1.45)</td>
<td>1.44 (1.12, 1.86)</td>
</tr>
<tr>
<td>Adjusted hazard rate ratio (95% CI)</td>
<td>1.00</td>
<td>0.86 (0.65, 1.15)</td>
<td>1.08 (0.82, 1.42)</td>
<td>1.01 (0.77, 1.34)</td>
<td>1.34 (1.02, 1.76)</td>
</tr>
</tbody>
</table>

1 Reference category.
2 Tests for linear trend were calculated by assigning the medians of intake in quintiles treated as a continuous variable in a Cox proportional hazard model.
3 SD (all such values).
4 Adjusted for age, sex, height, BMI, smoking, consumption of alcohol, total energy intake, systolic blood pressure, treatment for hypertension, total serum cholesterol, and level of education.
REFERENCES
Whole-grain intake and the prevalence of hypertriglyceridemic waist phenotype in Tehranian adults1–3

Ahmad Esmailzadeh, Parvin Mirmiran, and Fereidoun Azizi

ABSTRACT

Background: Although dietary guidelines recommend increased intake of grain products to prevent chronic diseases, no epidemiologic data associate whole-grain intake with hypertriglyceridemic waist (HW) phenotype.

Objective: We aimed to evaluate the relation between whole-grain intakes and the prevalence of HW phenotype in adults in Tehran, Iran.

Design: Whole-grain intake, serum triacylglycerol concentration, and waist circumference (WC) were assessed in a population-based, cross-sectional study of 827 Iranian subjects (357 men and 470 women) aged 18–74 y. HW phenotype was defined as serum triacylglycerol concentrations ≥150 mg/dL and concurrent WC ≥ 80 cm (men) and ≥79 cm (women).

Results: Mean (±SD) consumption of whole and refined grains was 93 ± 29 and 201 ± 57 g/d, respectively. Subjects in the highest quartile of whole-grain intake had a significantly lower prevalence of HW (29%) than did those in the lowest quartile (44%; \( P < 0.05 \)). Conversely, those in the highest quartile of refined-grain intake had a significantly higher prevalence of HW (45%) than did those in the lowest quartile (27%; \( P < 0.05 \)). After control for potential confounding factors, a significantly decreasing trend was observed for the risk of HW phenotype across quartiles of whole-grain intake (odds ratios among quartiles: 1.00, 0.95, 0.90, and 0.78, respectively; \( P \) for trend = 0.02). Higher consumption of refined grains was associated with better odds of HW phenotype (by quartile: 1.00, 1.38, 1.65, and 2.1; \( P \) for trend = 0.01).

Conclusion: Whole-grain intake is inversely and refined-grain intake is positively associated with the risk of HW. Am J Clin Nutr 2005;81:55–63.

KEY WORDS Whole grain, refined grain, waist circumference, serum triacylglycerol concentration, metabolic syndrome, cardiovascular risk factors

INTRODUCTION

Increasing evidence suggests that persons with metabolic syndrome are at increased risk of type 2 diabetes and cardiovascular disease (1, 2). The metabolic syndrome is defined as a pattern of metabolic disturbances including central obesity, insulin resistance, hyperglycemia, dyslipidemia, and hypertension (3). Although the precise prevalence of this syndrome is unknown, existing data suggest that the incidence is rising at an alarming rate (4, 5). In Tehran, Iran, it has been estimated to occur in >30% of adults (6), a prevalence significantly higher than that in most developed countries (7).

There is no globally accepted definition for the metabolic syndrome, and the World Health Organization (8), the European Group for the Study of Insulin Resistance (9), and the National Cholesterol Education Programme Adult Treatment Panel III (NCEP ATP III; 10) all support separate definitions. Many investigators assume, however, that insulin resistance is the fundamental metabolic defect underlying metabolic syndrome (3, 8, 10, 11). Insulin resistance in association with increased serum apolipoprotein B concentrations and high concentrations of small, dense LDL cholesterol in the serum has been called a metabolic triad (12) that could be identified by using an inexpensive screening tool called the hypertriglyceridemic waist (HW) phenotype (13).

Some investigators have reported that HW predicts the presence of the metabolic syndrome (14). Others recommended that index for the identification of a syndrome of lipid overaccumulation (15). Subjects with HW were nearly 4 times as likely to have angiographically defined coronary artery disease as were subjects who did not have the HW phenotype (13). Our previous studies showed that the HW phenotype is widespread in the urban population of Tehran, with an estimated prevalence of 19% in men (16) and 32% in women (17).

Few correlates of HW phenotype have been established. Although non-HDL cholesterol (18) and postprandial hyperlipidemia (19) were reported as metabolic factors that are related to HW phenotype, no evidence exists with respect to the dietary determinants of the phenotype. On the other hand, most published reports on the diet-disease relation have searched for the role of nutrients in chronic diseases (20, 21), and comparatively little emphasis was placed on the specific contribution of foods, especially whole-grain foods. Whole grains contain higher amounts of fiber, vitamin E, magnesium, antioxidants, and phytoestrogens than do non-whole-grain foods (22), and the protective effects of these nutrients against the risk of chronic diseases.
were shown by previous studies (23–26). Despite dietary recommendations for greater intakes of whole grains, little research has been conducted on the physiologic effects of a diet high in whole grains. This study was therefore conducted to assess the association between whole-grain consumption and HW phenotype in an urban population of Tehranian adults.

**SUBJECTS AND METHODS**

**Subjects**

This study was conducted within the framework of the Tehran Lipid and Glucose Study (TLGS), a prospective study performed in a representative sample of residents of District 13 of Tehran with the aims of ascertaining the prevalence of noncommunicable disease risk factors and of developing a healthy lifestyle to curtail these risk factors (27). In the TLGS, 15,005 persons aged ≥3 y were selected by random multistage cluster sampling. A representative sample of 1476 persons aged ≥3 y, including 861 subjects aged 18–74 y, was randomly selected for dietary assessment. In this population-based, cross-sectional study, subjects with a history of cardiovascular disease, diabetes, and stroke were excluded because of possible disease-related changes in their diets. We also excluded subjects whose reported daily energy intakes were < 800 kcal/d (3347 kJ/d) or > 4200 kcal/d (17,573 kJ/d) (28). These exclusions left 827 subjects (357 men and 470 women) aged 18–74 y, for the current analysis. The protocol for the study was approved by the research council of the Endocrine Research Center of Shaheed Beheshti University of Medical Sciences. Written informed consent was obtained from each subject.

**Assessment of dietary intake**

Usual dietary intake was assessed by using a 168-item semi-quantitative food-frequency questionnaire (FFQ). All the questionnaires were administered by trained dietitians who had ≥5 y of experience in the Nationwide Food Consumption Survey (29, 30). The FFQ consisted of a list of foods and a standard serving size for each (Willett format; 31). Participants were asked to report their frequency of consumption of a given serving of each food item during the previous year on a daily (eg, bread), weekly (eg, rice or meat), or monthly (eg, fish) basis. Portion sizes of consumed foods were converted to grams by using household measures (32). Each food and beverage was then coded according to the prescribed protocol and was analyzed for content of energy and the other nutrients with the use of NUTRITIONIST III software (version 7.0; N-Squared Computing, Salem, OR), which was designed for evaluation of Iranian foods.

We used a procedure developed by Jacobs et al (33) for classifying foods as whole or refined grains. Specifically, whole-grain foods included dark breads (eg, the Iranian breads sangak, barbari, and taftoon), barley bread, popcorn, cornflakes (in Iran, a whole-grain breakfast cereal), wheat germ, and bulgur. Refined grains included white breads (eg, the Iranian bread lavash and French bread), ice cream bread (ie, a refined-grain bread served with ice cream), noodles, pasta, rice, toasted bread, milled barley, sweet bread, white flour, starch, and biscuits.

The reliability of the FFQ in this cohort was evaluated in a randomly chosen subgroup of 132 subjects by comparing the nutrient consumption ascertained from their responses to the FFQ on 2 occasions. The correlation coefficients for the repeatability of white breads and dark breads were 0.85 and 0.89, respectively. The FFQ also had high reliability for nutrients. For example, the correlation coefficients were 0.81 for dietary fiber, 0.85 for magnesium, and 0.79 for vitamin E. Comparative validity was ascertained by comparison with intakes estimated from the average of twelve 24-h dietary recalls (one for each month of the year). Preliminary analysis of the validation study showed that nutrients commonly found in whole grains were moderately correlated between these 2 methods after control for total energy intake. The correlation coefficients were 0.69 for dietary fiber, 0.61 for vitamin E, and 0.67 for magnesium intake. The performance of the FFQ in assessing the intakes of individual grain products was good. For example, correlation coefficients between the FFQ and detailed dietary recalls were 0.63 for white breads and 0.71 for dark breads. Overall, these data indicate that the FFQ provides reasonably valid measurements of the average long-term dietary intakes.

**Assessment of other variables**

While the subjects were minimally clothed and not wearing shoes, weight was measured to the nearest 100 g by using digital scales. Height was measured by using a tape measure while the subject was in a standing position and not wearing shoes, and the shoulders were relaxed. Body mass index (BMI) was calculated as weight (in kg) divided by the height (in m) squared. A BMI ≥ 30 was considered to indicate obesity. By using an unstretched tape measure over light clothing and without any pressure to the body surface, waist circumference (WC) was measured at the narrowest level, and hip circumference was measured at the maximum level; measurements were recorded to the nearest 0.1 cm. Waist-to-hip ratio was calculated as WC divided by hip circumference. To avoid subjective error, all measurements were made by the same person (34).

Between 0700 and 0900, a blood sample was drawn into evacuated tubes from all study participants after 12–14-h overnight fasting. Blood samples were drawn while the subjects were in a sitting position according to the standard protocol, and the blood was centrifuged within 30–45 min after collection. All blood lipid analyses were done at the TLGS research laboratory on the day of blood collection. The analysis of samples was performed by using a Selectra 2 autoanalyzer (Vital Scientific, Spankeren, Netherlands). Serum triacylglycerol concentrations were assayed by using commercially available enzymatic reagents ( Pars Azmoon, Tehran, Iran) with glycerol phosphate oxidase. The performance of the assay was measured after every 20 tests by using the lipid control serums Percinorm (cat. no. 1446070; Boehringer Mannheim, Mannheim, Germany) and Perciopath (cat. no. 171778; Boehringer Mannheim) for normal and pathologic ranges of biochemical indexes, respectively. Lipid standard [cat. No. 759350 (calibrated for automated systems); Boehringer Mannheim] was used to calibrate the Selectra 2 autoanalyzer for each day of laboratory analysis. All samples were analyzed when internal quality control met the acceptable criteria. Interassay and intraassay CVs were 1.6% and 0.6% for triacylglycerol (35). Additional covariate information regarding age, smoking habits (36), physical activity (37), medical history, and current use of medications (36) was obtained by using validated questionnaires, as reported earlier.
Definition of hypertriglyceridemic waist

We used normative values of 80 cm for men and 79 cm for women as the threshold for an enlarged WC, as reported earlier (38). These cutoffs were optimal for predicting a risk factor ≥1 (ie, diabetes (fasting plasma glucose ≥ 126 mg/dL), hypertension (systolic blood pressure ≥ 140 mm Hg, diastolic blood pressure ≥ 90 mm Hg, or current use of antihypertensive medication), or dyslipidemia (based on NCEP ATP II guidelines; 39)) in the youngest adult participants (age category of 18–34 y) in the TLGS (38). We used the cutoffs of WC obtained from the youngest adults, because abdominal size increases with adult age (40), and thus the use as a threshold of values from any subpopulation but the youngest adults would be inappropriate (15). Cutoffs used in this study are considerably different from those used in the United States (15) and those recommended by the World Health Organization (41) because, as shown in our previous investigation(42), those cutoffs are inappropriate for Iranians.

For serum triacylglycerol concentrations, we used triacylglycerol ≥ 150 mg/dL as the cutoff, in accord with NCEP ATP III recommendations (43). Subjects were categorized in 4 phenotype groups on the basis of the mentioned cutoffs: 1) high serum triacylglycerol and high WC (men: triacylglycerol ≥ 150 mg/dL and WC ≥ 80 cm; women: triacylglycerol ≥ 150 mg/dL and WC ≥ 79 cm); 2) low serum triacylglycerol and high WC (men: triacylglycerol < 150 mg/dL and WC ≥ 80 cm; women: triacylglycerol < 150 mg/dL and WC ≥ 79 cm); 3) high serum triacylglycerol and low WC (men: triacylglycerol ≥ 150 mg/dL and WC < 80 cm; women: triacylglycerol ≥ 150 mg/dL and WC < 79 cm); and 4) low serum triacylglycerol and low WC (men: triacylglycerol < 150 mg/dL and WC < 80 cm; women: triacylglycerol < 150 mg/dL and WC < 79 cm).

Statistical analysis

SPSS software (version 9.05; SPSS Inc, Chicago) was used for all statistical analyses. In separate models, first-order interactions between sex and whole- and refined-grain intakes were entered to ascertain whether associations between men and women were similar. There was no significant effect of interactions by sex on the association of whole- and refined-grain intakes and HW phenotype. Cutoffs for quartiles of whole- and refined-grain intake were calculated, and subjects were categorized by quartiles: for whole grains, the cutoffs were <10, 10 to <71, 71 to <143, and ≥143 g/d for quartiles 1 through 4, respectively, and, for refined grains, the cutoffs were <125, 125 to <203, 203 to <281, and ≥281 g/d for quartiles 1 through 4, respectively. Significant differences in general characteristics across quartiles of whole- and refined-grain intake were searched by using one-way analysis of variance. If there was a significant main effect, Tukey’s test was used to detect pairwise differences. A chi-square test was used to detect significant differences in the distribution of subjects across quartiles of whole- and refined-grain intakes with regard to qualitative variables. We determined age-, sex-, and energy-adjusted means for dietary variables across quartiles of whole- and refined-grain intakes by using General Linear Mode. Analysis of covariance with the correction of Bonferroni was used to compare these means. All correlation coefficients reported were calculated as Pearson’s correlation coefficients. To ascertain the association of whole- and refined-grain intakes with HW phenotype, we used multivariable logistic regression models controlled for age (y), BMI, hip circumference (cm), energy intake (kcal/d), percentage of energy from fat, use of blood pressure medication (yes or no), cigarette smoking (ie, daily smokers, occasional smokers, former smokers, and never smokers), physical activity level (ie, light, moderate, and severe), and current estrogen replacement therapy among women (yes or no). When a significant association with whole- or refined-grain intakes was observed, we repeated the analysis after adjustment for intakes of fruit, vegetables, meat, and fish. In all multivariate models, the first quartile of whole- and refined-grain intakes was considered as a reference. The Mantel-Haenszel extension chi-square test was performed to assess the overall trend of an increasing quartile of whole- and refined-grain intakes associated with an increasing or decreasing likelihood of classification as a high-risk person.

Because the use of cutoffs for defining the HW phenotype implies a loss of information and because the association between WC and many diseases seems to be a continuous one, not a threshold association, we also studied relations between whole- and refined-grain intakes, WC, and serum triacylglycerol concentrations as continuous variables by using a multiple linear regression. Whole- and refined-grain intakes were both considered independent variables, and WC and serum triacylglycerol concentrations were considered dependent variables in separate models. All regression analyses were adjusted for age, BMI, hip circumference, energy intake, percentage of energy from fat, and intakes of fruit, vegetables, meat, and fish.

RESULTS

The reported mean daily intakes of whole and refined grains were 93 ± 29 g/d (men: 98 ± 36 g/d; women: 90 ± 24 g/d) and 201 ± 57 g/d (men: 206 ± 48; women: 197 ± 68 g/d), respectively. The food items that contributed most to whole-grain intakes were, in descending order, the barbari, taftoon and sangak breads; those that contributed most to refined grain intake were, also in descending order, rice, white breads, and biscuits.

Mean (±SD) age and anthropometric measurements as well as the distribution of subjects with regard to obesity, smoking, and physical activity across quartiles of whole- and refined-grains are shown in Table 1. Compared with participants in the lowest quartile, those in the highest quartile of whole-grain intake were older and had lower values of anthropometric measurements. Conversely, those in the lowest quartile of refined-grain intake had higher age, lower BMI, and lower WHR than did those in the highest quartile. There was no significant difference in WC across quartiles of refined-grain intake. Although most subjects in all quartiles of whole- and refined-grain intakes had light physical activity, there was a significant difference in the distribution of subjects across the quartiles with respect to physical activity levels. Subjects in the highest quartile of both whole- and refined-grain intakes were more likely to be daily smokers than were subjects in the other quartiles. The proportion of obese persons was lower among subjects in the highest quartile of whole-grain intakes and higher among those in the highest quartile of refined-grain intakes than it was among persons in the corresponding lowest quartiles.

The distribution of subjects by phenotypes of serum triacylglycerol concentration and WC across quartiles of whole- and refined-grain intakes is presented in Table 2. Subjects in the highest quartile of whole-grain intakes had a lower prevalence of the high triacylglycerol and high WC phenotype than did those in the lowest quartile. Conversely, this phenotype was more prevalent among
TABLE 1
Characteristics of Tehran Lipid and Glucose Study participants by quartiles of whole-grain and refined-grain intakes

<table>
<thead>
<tr>
<th></th>
<th>Whole-grain quartiles</th>
<th>Refined-grain quartiles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 (&lt;10 g/d)</td>
<td>2 (10 to &lt;71 g/d)</td>
</tr>
<tr>
<td>(n = 237)</td>
<td>(n = 176)</td>
<td>(n = 205)</td>
</tr>
<tr>
<td>Women (%)</td>
<td>58.8 ± 137</td>
<td>61.5 ± 13</td>
</tr>
<tr>
<td>Age (y)</td>
<td>36.4 ± 4.8</td>
<td>36.5 ± 4.8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.7 ± 4.9</td>
<td>25.8 ± 5.1</td>
</tr>
<tr>
<td>WHR</td>
<td>0.89 ± 0.08</td>
<td>0.87 ± 0.08</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>84 ± 12</td>
<td>84 ± 13</td>
</tr>
<tr>
<td>Physical activity level (%)</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Light</td>
<td>62.0 ± 55</td>
<td>62.0 ± 55</td>
</tr>
<tr>
<td>Moderate</td>
<td>23.0 ± 31</td>
<td>23.0 ± 31</td>
</tr>
<tr>
<td>Heavy</td>
<td>15.0 ± 13</td>
<td>11.0 ± 11</td>
</tr>
<tr>
<td>Current daily smokers (%)</td>
<td>12.0 ± 7</td>
<td>7.0 ± 15</td>
</tr>
<tr>
<td>Obese (%)</td>
<td>22.0 ± 20</td>
<td>19.0 ± 18</td>
</tr>
<tr>
<td>Current estrogen use (%)</td>
<td>14.0 ± 15</td>
<td>15.0 ± 15</td>
</tr>
</tbody>
</table>

* WHR, waist-hip ratio.

1 For differences among whole-grain categories by using ANOVA with Tukey’s test for data that are means ± SDs and chi-square for data that are percentages.

2 For differences among refined-grain categories by using ANOVA with Tukey’s test for data that are means ± SDs and chi-square for data that are percentages.

3 In women only.

Those in the highest quartile of refined-grain intakes than among those in the lowest quartile. As whole-grain intakes increased, the proportion of subjects with the low triacylglycerol and low WC phenotype increased. Conversely, as refined-grain intakes increased, the proportion of subjects with the low triacylglycerol and low WC phenotype decreased. The prevalence of high triacylglycerol and low WC and of low triacylglycerol and high WC phenotypes did not differ significantly across quartiles of whole- and refined-grain intakes.

Age-, sex-, and energy-adjusted means for dietary variables across quartiles of whole- and refined-grain intakes are presented in Table 3. Higher intakes of whole grains were associated with a healthier diet: subjects in the highest quartile also consumed less cholesterol and meat and more dietary fiber, fruit, and vegetables than did those in the lowest quartile. The intakes of whole grains were positively associated with total intakes of dietary fiber (r = 0.43), magnesium (r = 0.51), and vitamin B-6 (r = 0.48), which are important constituents of whole grains.

Multivariate-adjusted odds ratios for the HW phenotype (high triacylglycerol and high WC) compared with the low triacylglycerol and low WC phenotype across quartiles of whole- and refined-grain intakes are shown in Figure 1. After adjustment for

TABLE 2
Prevalence of different phenotypes of serum triacylglycerol concentration and waist circumference (WC) across quartiles of whole- and refined-grain intakes

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Whole-grain quartiles</th>
<th>Refined-grain quartiles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 (&lt;10 g/d)</td>
<td>2 (10 to &lt;71 g/d)</td>
</tr>
<tr>
<td>(n = 237)</td>
<td>(n = 176)</td>
<td>(n = 205)</td>
</tr>
<tr>
<td>High triacylglycerol and high WC (%)</td>
<td>44.0 ± 36</td>
<td>36.0 ± 31</td>
</tr>
<tr>
<td>High triacylglycerol and low WC (%)</td>
<td>5.0 ± 4</td>
<td>5.0 ± 6</td>
</tr>
<tr>
<td>Low triacylglycerol and high WC (%)</td>
<td>32.0 ± 35</td>
<td>33.0 ± 32</td>
</tr>
<tr>
<td>Low triacylglycerol and low WC (%)</td>
<td>19.0 ± 25</td>
<td>31.0 ± 33</td>
</tr>
</tbody>
</table>

* Cutoffs in parentheses.

1 For differences among whole-grain categories (chi-square test).

2 For differences among whole-grain categories (chi-square test).

3 For differences among refined-grain categories (chi-square test).

4 Triacylglycerol ≥ 150 mg/dL plus WC ≥ 80 cm (men) and WC ≥ 79 cm (women).

5 Triacylglycerol ≥ 150 mg/dL plus WC < 80 cm (men) and WC < 79 cm (women).

6 Triacylglycerol ≥ 150 mg/dL plus WC ≥ 80 cm (men) and WC ≥ 79 cm (women).

7 Triacylglycerol < 150 mg/dL plus WC < 80 cm (men) and WC < 79 cm (women).
potential confounding variables and dietary factors associated with diets high in whole grains, a significantly decreasing trend for HW phenotype was observed among whole-grain quartiles (A). Higher consumption of refined grains was associated with higher metabolic risk factors. Multivariate adjusted models showed that subjects in the highest quartile of refined-grain intakes had a greater chance of HW phenotype than did those in the lowest quartile (B). There was a significantly increasing trend for HW phenotype across refined-grain quartiles.

The results of simultaneously entering whole- and refined-grain intakes to predict WC and serum triacylglycerol concentrations after adjustment for age, BMI, hip circumference, energy intake, percentage of energy from fat, and intakes of fruit, vegetables, meat, and fish are shown in Table 4. Both whole- and refined-grain intakes were independently related to serum triacylglycerol concentrations. The association with whole-grain intakes was negative, and that with refined-grain intakes was positive. There was a significant inverse association between whole-grain intakes and WC, but the association with refined-grain intakes was not significant.

**DISCUSSION**

The current study, conducted in part of the urban population of Tehran, showed a favorable inverse association of whole-grain intakes with HW phenotype. In contrast, refined-grain intakes were associated with better odds of HW phenotype. To our knowledge, this is the first study reporting the association between whole-grain intakes and HW phenotype.

A favorable association of whole-grain consumption with HW phenotype may be attributed to the healthy lifestyle associated with higher intakes of whole grains. However, the apparently protective effect of whole-grain consumption persisted in multivariate models. Moreover, some intermediary events, including dyslipidemia or hypertension, could have led to changes in diet and may therefore confound the association between whole-grain intakes and metabolic risks. However, any confounding effects from these indications would tend to attenuate the protective effect of whole-grain intake because the tendency would be for subjects to increase their intake of whole-grain foods if they perceived themselves to be at an elevated risk of chronic diseases.

Although the risk of the atherogenic metabolic triad (ie, hyperinsulinemia, hyperapolipoprotein B, and small, dense LDL) could be identified by using an inexpensive screening tool that included the simultaneous measurement of WC and fasting serum triacylglycerol concentrations (13), relatively few studies have examined the predictors of HW phenotype (18, 19), and there is no evidence in current literature on the dietary determinants of this phenotype. In the current study, the prevalence and odds of HW phenotype were lower in subjects with higher intakes of whole-grain foods. Our findings are in line with recent studies reporting the health benefits of whole-grain intakes. Our
previous investigation showed a favorable association of whole-grain intakes with metabolic syndrome (44). McKeown et al (45) also showed a lower prevalence and lower odds of insulin resistance syndrome in subjects in the highest quintile of whole-grain intakes than in those in the lowest quintile. Investigators of other epidemiologic studies also came to the conclusion that higher consumption of whole grains protects against most noncommunicable diseases (46, 47). An interventional crossover study also supports the hypothesis that diets rich in whole-grain foods are associated with lower insulin concentrations (48).

The biological mechanisms whereby whole-grain foods may exert their protective effects, although not clear, are likely to be many. Greater intakes of many constituents of whole grains, including dietary fiber, vitamin E, folate, and magnesium, have been independently associated with reduced metabolic risk. Even after adjustment for these components of whole-grain foods by Liu et al (49), a significant inverse relation of whole-grain intakes to metabolic risks was still evident, which suggests an additional protective effect of other constituents or their interactions. McKeown et al (45) reported that fiber from cereals was inversely related to the prevalence of metabolic syndrome, whereas fiber from fruit and vegetables was not. Observational data also indicated that fiber from cereals provides greater protection against diabetes than does fiber from other sources (50, 51). Adjustment for cereal fiber in the study of McKeown et al (45) weakened the associations between whole-grain intakes and metabolic syndrome, which suggests that that relation may be due in part to fiber or factors related to fiber. In general, because of their physical form and viscous fiber content, whole-grain products tend to be digested slowly and absorbed, and thus they have relatively low glycemic indexes. In some metabolic studies of both diabetic and nondiabetic subjects, high intakes of low-glycemic-index foods have been associated with lower concentrations of LDL and glycated hemoglobin and lower amounts of urinary C-peptide excretion (52, 53).

In the current study, higher consumption of refined grains was associated with better odds of HW phenotype. Previous studies showed a positive association between refined-grain intakes and the risk of type 2 diabetes (54) and metabolic syndrome (44). Other investigators found no evidence for the association between refined-grain intakes and metabolic risk factors (47). This lack of evidence may be explained by differences in the glycemic

### TABLE 4

<table>
<thead>
<tr>
<th></th>
<th>Whole-grain intake</th>
<th>Refined-grain intake</th>
<th>Percentage of variance explained$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference</td>
<td>$-0.413 \pm 0.117$</td>
<td>$0.227 \pm 0.093$</td>
<td>43.4</td>
</tr>
<tr>
<td>Serum triacylglycerol</td>
<td>$-0.561 \pm 0.195$</td>
<td>$0.429 \pm 0.163$</td>
<td>51.4</td>
</tr>
</tbody>
</table>

$^1$ Variance explained by whole-grain intake, refined-grain intake, age, BMI, hip circumference, energy intake, percentage of energy from fat, and intakes of fruit, vegetables, meats, and fish.

$^2$ SEM (all such values).

$^3$ These values are significant at $P < 0.05$. 

**FIGURE 1.** Multivariate-adjusted odds ratios and 95% CIs for hypertriglyceridemic waist (HW) phenotype [high triacylglycerol and high waist circumference (WC)] compared with low triacylglycerol and low WC across quartiles 2 (●), 3 (□), and 4 (▲) of whole- and refined-grain intakes. HW phenotype: triacylglycerol concentrations ≥ 150 mg/dL and WC ≥ 80 cm (men) and triacylglycerol ≥ 150 mg/dL and WC ≥ 79 cm (women); low triacylglycerol and low WC: triacylglycerol concentrations < 150 mg/dL and WC < 80 cm (men) and triacylglycerol < 150 mg/dL and WC < 79 cm (women). Cutoffs for quartiles of whole-grain intakes were < 10, 10 to < 71, 71 to < 143, and ≥ 143 g/d for quartiles 1 through 4, respectively. Cutoffs for quartiles of refined-grain intakes were < 125, 125 to < 203, 203 to < 281, and ≥ 281 g/d for quartiles 1 through 4, respectively. The presented odds ratios were adjusted for age, BMI, hip circumference, energy intake, percentage of energy from fat, use of blood pressure medication, cigarette smoking, physical activity level (all subjects), and current estrogen replacement therapy (among women). After adjustment for potential confounding variables, a significant, decreasing trend for HW was observed among whole-grain quartiles (A). Subjects in the highest quartile of refined-grain intakes were more likely to have the HW phenotype (B). There was a significantly increasing trend for HW phenotype across refined-grain quartiles.
indexes of various refined-grain foods. A high dietary glycemic index has been proposed to be associated with higher metabolic risk (45). High-glycemic-index foods produce higher postprandial blood glucose concentrations than do low-glycemic-index foods, and, in the long term, the former will generate a greater demand for insulin (52, 55).

The Food Guide Pyramid of the US Department of Agriculture recommends the consumption of 6–11 servings of grain products per day, but the amount of whole grains is not specified. The National Food Consumption Survey (30) showed that intakes of whole grains by Iranians were lower than that recommendation (<2 servings/d). This amount is higher than the 0.5 serving/d consumed by Americans (56) and lower than the amount consumed by the participants in the current study, in which subjects in the highest quartile of whole-grain intakes consumed 229 g whole grains/d (≈5.5 servings/d). Considering the favorable association of whole-grain consumption to HW in the current study, it seems that the recommended greater consumption of these products is likely to have significant benefits in reducing the risk of metabolic disorder.

Several limitations should be considered when examining the results of this study. First, we used Jacobs’s definition for categorizing grain products. This definition is less stringent than the one used by the Food and Drug Administration (57), and it has some limitations. For example, it does not allow comparison: if 2 food items have the same percentage of whole grain, that does not necessarily mean that they have the same grain structure or the same components. Second, we used cross-sectional data to identify the association between whole- and refined-grain intakes and the HW phenotype. Future studies using longitudinal data will provide stronger evidence on this relation. Third, because of the fixed food categories associated with the FFQ, it is difficult to accurately separate whole- and refined-grain foods from some other foods. For example, the category of dark breads, such as tahnoon, may include breads made with refined grain. Yet, despite this potential measurement error in exposure, which would tend to attenuate the associations, we found significant associations between whole-grain intakes and HW phenotype. As previous studies showed, however, the FFQ tends to underestimate refined-grain intakes (58), and that could diminish the associations observed between exposure and outcome (59). Despite this, we found significant associations between refined-grain intakes and HW phenotype. Fourth, diets rich in whole-grain foods appear to reflect an overall healthier lifestyle that may not have been accurately captured and controlled for in our analysis, which may have resulted in residual confounding. Fifth, subjects with known coronary artery disease, diabetes, and stroke were excluded from the study. These exclusions may have reduced the likelihood of finding the most significant trends in the odds of HW phenotype across quartiles of whole- and refined-grain consumption. Sixth, in the TLGS, WC was measured at the point of noticeable waist narrowing, which may have resulted in lower WC values than might be obtained by using other common sites of measurement. Whereas the World Health Organization Expert Committee on Physical Status (60) recommended waist measurement midway between the lower rib and the iliac crest, the third National Health and Nutrition Examination Survey guidelines (61) prescribed measurement at a point just above the right ilium, and the recommendation of the North American Association for the Study of Obesity and the National Heart, Lung, and Blood Institute (62) is to measure at the right iliac crest. The lack of standard measurement for WC is unfortunate and makes comparison with other studies difficult. It is believed that the use of the narrowest waist measurement offers greater ease of acceptance and interpretation by the public and may facilitate self-measurement in addition to clinical use. The use of the narrowest point for waist circumference probably explains why the optimal thresholds for men and women differ by just 1 cm in our population.

This study also has several strengths. Using a sample that was representative of the overall population of Tehran, we found a cross-sectional relation between whole- and refined-grain intakes and HW phenotype. In addition, the use of logistic regression models in this study allowed for simultaneous adjustment of confounding variables in the association of whole- and refined-grain intakes with HW phenotype.

We conclude that whole-grain intakes are inversely and refined-grain intakes are directly associated with HW phenotype. Therefore, efforts should be made to reduce the cost and increase the availability and consumption of whole-grain products. Sustained over time, such developments have the potential to substantially reduce the incidence of HW phenotype, which in turn would reduce the incidence of athrogenic metabolic triad and, possibly, other chronic diseases.

We thank the participants of the Tehran Lipid and Glucose Study for their enthusiastic support and the staff of the Endocrine Research Center, Tehran Lipid and Glucose Study unit, for their valuable help in conducting this study. AE and PM designed the study, collected and analyzed the data, and wrote the manuscript. FA supervised the research. None of the authors had any personal or financial conflicts of interest.

REFERENCES


Rice bran oil, not fiber, lowers cholesterol in humans

Marlene M Most, Richard Tulley, Silvia Morales, and Michael Lefevre

ABSTRACT

Background: The cholesterol-lowering abilities of rice bran’s fiber and oil apart from its fatty acid composition remain unclear.

Objective: The objective of the study was to assess the effects of defatted rice bran and rice bran oil in an average American diet on blood lipids in moderately hypercholesterolemic persons.

Design: Study 1 used a parallel-arm design. Twenty-six healthy volunteers consumed a diet with 13–22 g dietary fiber/d for 3 wk, and then 13 of the volunteers were switched to a diet with defatted rice bran to double the fiber intake for 5 wk. Study 2 was a randomized, crossover, 10-wk feeding study performed in 14 volunteers who consumed a diet with rice bran oil (1/3 of the total dietary fat) substituted for an oil blend that had a fatty acid composition similar to that of the rice bran oil. Serum lipids and factor VII were measured in both studies.

Results: Defatted rice bran did not lower lipid concentrations. In study 2, total cholesterol was significantly lower with consumption of the diet containing rice bran oil than with consumption of the control diet. Moreover, with consumption of the rice bran oil diet, LDL cholesterol decreased by 7% (P < 0.0004), whereas HDL cholesterol was unchanged.

Conclusions: Rice bran oil, not fiber, lowers cholesterol in healthy, moderately hypercholesterolemic adults. There were no substantial differences in the fatty acid composition of the diets; therefore, the reduction of cholesterol was due to other components present in the rice bran oil, such as unsaponifiable compounds. Am J Clin Nutr 2005;81:64–8.

KEY WORDS Rice bran fiber, rice bran oil, lipoproteins, phytosterols

INTRODUCTION

Rice bran, a coproduct of milled rice, and its oil may have cardiovascular health benefits. Human consumption of rice bran has been limited, primarily because of the rapid onset of rancidity in rice bran, but methods to stabilize rice bran and to extract its oil have been developed. Interest in rice bran grew from the determination that the inclusion of oat bran in the diet lowers serum cholesterol (1, 2). Studies of rice bran supplementation in humans have been developed. Interest in rice bran oil apart from its fatty acid composition remain unclear.

Believed that rice bran lowers cholesterol by a mechanism different from that of oat bran. Decreases in cholesterol were found in hypercholesterolemic subjects who replaced their usual cooking oils with rice bran oil (9) and in middle-aged and elderly subjects consuming a low-fat diet containing rice bran oil (10). Yet rice bran oil typically contains 20% saturated fatty acids and approximately equal amounts of oleic and linoleic fatty acids (11). Previous research showed the deleterious effects of saturated fatty acids on total cholesterol concentrations, and the fact that rice bran oil lowers cholesterol is contrary to these findings. Research now suggests that rice bran oil’s cholesterol-lowering properties are explained by its unsaponifiable components more than by its fatty acid composition (12, 13). Attention has begun to focus on the components of rice bran oil, including phytosterols, triterpene alcohols, tocopherols, and tocotrienols, as possible hypcholesterolemic agents.

We examined further the cholesterol-lowering abilities of rice bran’s fiber and oil apart from its fatty acid composition. This was accomplished with 2 well-controlled feeding studies designed to evaluate the effects of using defatted rice bran and rice bran oil in an average American diet on cardiovascular disease risk factors in men and women.

SUBJECTS AND METHODS

Subjects

For study 1, 27 healthy men and women were recruited in 2 cohorts. One woman was taking hormone replacement therapy, and the other women were premenopausal and not taking oral contraceptives. One man was dropped from the study after random assignment to the treatment diet because of an allergic reaction. Fourteen healthy men and premenopausal women (3 taking oral contraceptives) participated in study 2. The subjects’ characteristics are shown in Table 1.

Eligible subjects were 18–50 y old and had total serum cholesterol concentrations between the 25th and 90th percentiles after adjustment for sex, age, and race (14); triacylglycerol concentrations < 90th percentile after adjustment for sex, age, and...
race; LDL concentrations < 4.91 mmol/L (190 mg/dL) and HDL concentrations ≥ 0.65 mmol/L (25 mg/dL); and a body mass index (BMI; in kg/m²) ≥ 30. Exclusion criteria included renal, hepatic, cardiovascular, endocrine, gastrointestinal, or other systemic disease; hypertension; pregnancy (for women); history of drug or alcohol abuse; smoking or other tobacco use; chronic use of prescribed medication; extreme dietary habits such as vegetarianism or severely low fat intakes; multiple food allergies; of prescribed medication; extreme dietary habits such as vegetarianism or severely low fat intakes; multiple food allergies; multiple food allergies; and current weight-loss efforts.

The study protocol and consent form were approved by the Louisiana State University’s Institutional Review Board. Written informed consent was obtained from all subjects, and all subjects received monetary compensation for their participation.

**Experimental design**

**Study 1: defatted rice bran**

All subjects began with consumption of a run-in diet for 3 wk and then were randomly assigned to either the control or intervention diet for an additional 5 wk. Assessment of outcome measures occurred at the end of the run-in period and at the end of the study. Blood samples were collected in triplicate on separate days to minimize the influence of biologic variability in these measures. Body weight was measured twice a week, and energy was adjusted to ensure weight stability.

The study 1 diets were a low-fiber control diet (control 1) and a high-fiber intervention diet containing defatted rice bran (DRB). The control diet provided 13–22 g dietary fiber/d, varying with total energy, whereas the addition of DRB (56–94 g/d, varying with total energy level) to the intervention diet doubled the fiber content. Both diets provided 37% of total energy as fat. DRB was incorporated into muffins, cookies, and breads. A 4-d menu rotation was used to maintain variety throughout the study. The macronutrient composition of each diet (an average of the 4 menu plans, each at 2 different energy amounts), as determined by the Pennington Center’s Food Analysis Laboratory, is shown in Table 2.

**Study 2: rice bran oil**

Study 2 used a randomized, double-blind, crossover design with two 5-wk diet periods. Assessment of outcome measures occurred at the end of each diet period. Blood samples were collected in triplicate on separate days to minimize the influence of biologic variability in these measures. Body weight was measured twice a week, and energy was adjusted to ensure weight stability.

To determine whether unsaponifiable components present in rice bran oil (RBO) affect lipid metabolism, the fatty acid composition of RBO was matched with that of an oil blend that was used in the control diet. The fatty acid profile of the RBO that was obtained for the second feeding study was determined. Then other oils were combined, chemically analyzed, and adjusted until the best match of the RBO’s fatty acid profile was achieved. The oil blend was composed of peanut oil, olive oil, corn oil, canola oil, palm oil, and butter. The comparison of the control blend and RBOS for the major fatty acids and for the tocopherol, tocotrienol, and oryzanol contents is shown in Table 3.

Both diets were designed to provide 37% of total energy as fat. For the study 2 control diet (control 2), one-third of the total dietary fat was in the form of the oil blend, and for the RBO intervention diet, the oil blend was replaced with RBO. The oil blend or rice bran oil was incorporated into recipes for a 5-d menu rotation. Other fats were added to the diets so that the total diet would provide 15%, 17%, and 6% of energy as saturated, monounsaturated, and polyunsaturated fat, respectively. The total dietary cholesterol was ~125 mg/1000 kcal. To keep the participants blinded to their diet assignment, the control 2 (oil blend) and the control 1 (rice bran oil) diets were blinded.

---

**TABLE 1**

Baseline characteristics at screening of subjects in study 1 and study 2

<table>
<thead>
<tr>
<th></th>
<th>Study 1 (n = 13 F, 13 M)</th>
<th>Study 2 (n = 7 F, 7 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>32.9 ± 1.7</td>
<td>33.6 ± 2.8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.3 ± 0.6</td>
<td>24.8 ± 0.7</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.03 ± 0.12</td>
<td>5.33 ± 0.11</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.29 ± 0.08</td>
<td>1.19 ± 0.07</td>
</tr>
<tr>
<td>LDLC cholesterol (mmol/L)</td>
<td>3.33 ± 0.15</td>
<td>3.65 ± 0.12</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/L)</td>
<td>0.84 ± 0.08</td>
<td>1.00 ± 0.09</td>
</tr>
</tbody>
</table>

1 All values are x ± SEM of subject’s average of replicate measurements. To convert cholesterol values to mg/dL, divide by 0.02586; to convert triacylglycerols values to mg/dL, divide by 0.01129.

---

**TABLE 2**

Chemical composition of the diets provided to subjects participating in study 1

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>Defatted rice bran diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat (% of energy)</td>
<td>Total</td>
<td>36.6 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Saturated</td>
<td>14.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Polysaturated</td>
<td>10.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Monounsaturated</td>
<td>11.4 ± 0.4</td>
</tr>
<tr>
<td>Carbohydrate (% of energy)</td>
<td>51.3 ± 1.4</td>
<td>50.6 ± 1.3²</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>12.1 ± 0.6</td>
<td>12.9 ± 0.5²</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>16.6 ± 1.8</td>
<td>33.3 ± 4.7²</td>
</tr>
</tbody>
</table>

1 All values are x ± SEM and reflect the average values of 4 menu plans at 2 energy levels (9205 and 12 552 kJ; 2200 and 3000 kcal).

²,³ Significantly different from control diet (two-sample t test): ²P < 0.05, ³P < 0.002.

---

**TABLE 3**

Comparison of the fatty acid profile and of the tocopherol, tocotrienol, and oryzanol content of the control oil blend and the rice bran oil used in study 2, as determined by chemical analysis

<table>
<thead>
<tr>
<th></th>
<th>Control oil blend</th>
<th>Rice bran oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0 (g/100 g)</td>
<td>0.37</td>
<td>0.40</td>
</tr>
<tr>
<td>16:0 (g/100 g)</td>
<td>12.96</td>
<td>14.60</td>
</tr>
<tr>
<td>18:0 (g/100 g)</td>
<td>2.97</td>
<td>2.09</td>
</tr>
<tr>
<td>18:1 (g/100 g)</td>
<td>45.43</td>
<td>44.51</td>
</tr>
<tr>
<td>18:2 (g/100 g)</td>
<td>35.90</td>
<td>36.59</td>
</tr>
<tr>
<td>18:3n (g/100 g)</td>
<td>0.84</td>
<td>0.87</td>
</tr>
<tr>
<td>α-Tocopherol (µg/g)</td>
<td>108.4</td>
<td>180.0</td>
</tr>
<tr>
<td>α-Tocotrienol (µg/g)</td>
<td>34.4</td>
<td>218.0</td>
</tr>
<tr>
<td>γ-Tocopherol (µg/g)</td>
<td>127.5</td>
<td>38.0</td>
</tr>
<tr>
<td>γ-Tocotrienol (µg/g)</td>
<td>11.7</td>
<td>59.0</td>
</tr>
<tr>
<td>δ-Tocopherol (µg/g)</td>
<td>2.92</td>
<td>—</td>
</tr>
<tr>
<td>δ-Tocotrienol (µg/g)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Oryzanol (mg/g)</td>
<td>0.04</td>
<td>15.8</td>
</tr>
</tbody>
</table>
TABLE 4
Chemical composition of the diets provided to subjects participating in study 2

<table>
<thead>
<tr>
<th></th>
<th>Control oil blend diet</th>
<th>Rice bran oil diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat (% of energy)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>38.4 ± 0.5</td>
<td>37.8 ± 0.3</td>
</tr>
<tr>
<td>Saturated</td>
<td>15.6 ± 0.2</td>
<td>15.7 ± 0.2</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>7.0 ± 0.2</td>
<td>6.7 ± 0.1</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>15.8 ± 0.2</td>
<td>15.7 ± 0.2</td>
</tr>
<tr>
<td>Carbohydrate (% of energy)</td>
<td>47.1 ± 0.4</td>
<td>47.5 ± 0.5</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>14.5 ± 0.3</td>
<td>14.8 ± 0.3</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>320.9 ± 14.6</td>
<td>366.4 ± 20.0†</td>
</tr>
</tbody>
</table>

† All values are x ± SEM and reflect the average values of 5 menu plans at 2 energy levels (9205 and 12 552 kJ; 2200 and 3000 kcal).

Results

Study 1: defatted rice bran

Of the 27 subjects enrolled, 26 completed the entire study. The one subject who dropped out had only run-in period data, which was not taken into account in the analysis. The mean (±SEM) age of the subjects was 32.9 ± 1.7 y, and their mean BMI was 24.3 ± 0.6 (Table 1). Body weight did not change during the study (data not shown). All participants consumed the control diet during run-in and thus are sampled from one population. They subsequently were randomly assigned to the separate treatment groups—control or DRB. Thus, the fixed-effects model included the end-of-run-in level of response variable as a covariate to account for any possible differences that might have existed. On the basis of this model, lipid and lipoprotein concentrations and factor VII activity in response to the diets are shown in Table 5. Unexpectedly, after 5 wk, LDL cholesterol and apolipoprotein B were higher in the subjects consuming the DRB than in those consuming the control diet. Despite the change in LDL cholesterol, total cholesterol was not significantly changed by either diet. All other cardiovascular disease risk factors that we measured were unchanged.

Study 2: rice bran oil

At enrollment, the average age of the subject was 33.6 ± 2.8 y, and the average BMI was 24.8 ± 0.7 (Table 1). At enrollment, the subjects’ total cholesterol was 5.33 ± 0.11 mmol/L, HDL was 1.19 ± 0.09 mmol/L, and triacylglycerol was 1.12 mmol/L. All subjects enrolled completed the study.

Lipid and apolipoprotein concentrations and factor VII activity in response to the 2 diets are shown in Table 6. There was a significant effect of diet on total cholesterol, LDL cholesterol, and apolipoprotein B. Total cholesterol was lowest on the diet containing RBO, because of the lower concentration of LDL cholesterol; HDL cholesterol did not change. Triacylglycerol and factor VII were unchanged.

Discussion

Evidence from these 2 well-controlled feeding studies shows that it is the RBO, and not the fiber, that lowers blood lipids in men and women with borderline high total cholesterol. Rice bran
TABLE 5
Lipid and apolipoprotein concentrations and factor VII activity in study 1

<table>
<thead>
<tr>
<th></th>
<th>Run-in diet</th>
<th>Control diet</th>
<th>Defatted rice bran diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 26)</td>
<td>(n = 13)</td>
<td>(n = 13)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.01 ± 0.12</td>
<td>4.84 ± 0.17</td>
<td>5.21 ± 0.26</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.32 ± 0.08</td>
<td>1.44 ± 0.14</td>
<td>1.22 ± 0.09</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>3.25 ± 0.13</td>
<td>3.04 ± 0.16</td>
<td>3.53 ± 0.25</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/L)</td>
<td>0.93 ± 0.08</td>
<td>0.76 ± 0.09</td>
<td>0.99 ± 0.12</td>
</tr>
<tr>
<td>Apolipoprotein A-1 (g/L)</td>
<td>1.36 ± 0.05</td>
<td>1.43 ± 0.11</td>
<td>1.32 ± 0.05</td>
</tr>
<tr>
<td>Apolipoprotein B (g/L)</td>
<td>0.95 ± 0.04</td>
<td>0.88 ± 0.05</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>Factor VII (%)</td>
<td>81.69 ± 3.31</td>
<td>80.92 ± 5.58</td>
<td>85.49 ± 5.23</td>
</tr>
</tbody>
</table>

\(^1\) All values are \(\bar{x} \pm SEM\) of each subject’s average of replicate measurements taken at end of diet periods. To convert cholesterol values to mg/dL, divide by 0.02586; to convert triacylglycerol values to mg/dL, divide by 0.01129.

\(^2\) Significantly different from control diet (two-sided \(t\) tests for pairwise comparison): \(^2P = 0.0204, \(^4P = 0.0299.\)

\(^3\) Statistical analyses performed on log-transformed values; no significant difference was found.

TABLE 6
Lipid and apolipoprotein concentrations and factor VII activity at the end of study 2

<table>
<thead>
<tr>
<th></th>
<th>Control oil blend diet</th>
<th>Rice bran oil diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 14)</td>
<td>(n = 14)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.22 ± 0.15</td>
<td>4.95 ± 0.14(^4)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.22 ± 0.06</td>
<td>1.22 ± 0.07</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>3.57 ± 0.15</td>
<td>3.30 ± 0.14(^4)</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/L)</td>
<td>0.93 ± 0.11</td>
<td>0.93 ± 0.08</td>
</tr>
<tr>
<td>Apolipoprotein A-1 (g/L)</td>
<td>1.32 ± 0.05</td>
<td>1.34 ± 0.05</td>
</tr>
<tr>
<td>Apolipoprotein B (g/L)</td>
<td>1.03 ± 0.05</td>
<td>0.97 ± 0.04(^4)</td>
</tr>
<tr>
<td>Factor VII (%)</td>
<td>103.48 ± 3.66</td>
<td>101.48 ± 4.38</td>
</tr>
</tbody>
</table>

\(^1\) All values are \(\bar{x} \pm SEM\) of each subject’s average of replicate measurements. To convert cholesterol values to mg/dL, divide by 0.02586; to convert triacylglycerol values to mg/dL, divide by 0.01129.

\(^2\) Significantly different from oil blend diet (\(F\) tests using a mixed model): \(^2P = 0.0036, \(^3P = 0.0004, \(^5P = 0.0054.\)

\(^3\) Statistical analyses performed on log-transformed values.

Other investigators of rice bran have implicated the unsaponifiable compounds as being responsible for its cholesterol-lowering properties. The amounts present in commercial RBO are dependent on the refining process (17). The most notable compound is \(\gamma\)-oryzanol, a ferulate ester of triterpene alcohols (12). Major components of the triterpene alcohols are cycloartenol and 24-methylene cycloartenol. Also notable are the phytosterols campsternol and \(\beta\)-sitostanol, which are found at relatively high amounts in RBO. When the plant sterols from RBO were incorporated into margarine and provided at 2.1 g/d to normolipidemic men and women, total cholesterol decreased by 5% and LDL cholesterol decreased by 9% (18). The investigators postulated that the effect was due to the \(\beta\)-sitostanol and other 4-desmethylsterols and not to the 4,4'-dimethylsterols, such as cycloartenol and 24-methylene cycloartenol. The \(\beta\)-sitostanol structure is more similar to that of cholesterol than is that of the 4,4'-dimethylsterols, and it may be more effective than the 4,4'-dimethylsterols in inhibiting cholesterol absorption in the small intestine. This is further supported by Weststrate and Meijer (19),
who found no effect on cholesterol concentrations of an RBO margarine that contained more 4,4′-dimethylsterols and less 4-desmethylolesterols.

RBO also is rich in tocotrienols; the major components are the \( \beta \)- and \( \gamma \)-tocotrienols (12). It is postulated that tocotrienols, especially \( \gamma \)-tocotrienols, lower cholesterol through the inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in endogenous cholesterol synthesis (20). Two studies have reported that a tocotrienol-rich fraction of rice bran, when taken in combination with an American Heart Association Step 1 diet, lowers serum total and LDL-cholesterol concentrations in hypercholesterolemic persons (21, 22). However, these effects of tocotrienol have been questioned, and confirmation is needed (20).

Our results confirm previous findings of the total and LDL-cholesterol-lowering effects of RBO in humans. By matching the fatty acids of the RBO with a control oil blend, we showed that the effect of RBO on serum cholesterol concentrations is due to the unsaponifiables present in it and not to its fatty acid profile. RBO that contains these compounds could become an important functional food with cardiovascular health benefits.

We thank the volunteers who graciously gave their time and effort to these studies. We also thank our clinic coordinator, Susan Mancuso, and dietitian coordinator, Stephanie Schorle.

MMM, RT, and ML designed the study; MMM and RT collected the data, and ML provided advice; SM performed the statistical analysis; and MMM wrote the manuscript with input from the other authors. None of the authors had a conflict of interest.

REFERENCES

Alternate-day fasting in nonobese subjects: effects on body weight, body composition, and energy metabolism

Leonie K Heilbronn, Steven R Smith, Corby K Martin, Stephen D Anton, and Eric Ravussin

ABSTRACT

Background: Prolonged dietary restriction increases the life span in rodents. Some evidence suggests that alternate-day fasting may also prolong the life span.

Objective: Our goal was to determine whether alternate-day fasting is a feasible method of dietary restriction in nonobese humans and whether it improves known biomarkers of longevity.

Design: Nonobese subjects (8 men and 8 women) fasted every other day for 22 d. Body weight, body composition, resting metabolic rate (RMR), respiratory quotient (RQ), temperature, fasting serum glucose, insulin, free fatty acids, and ghrelin were assessed at baseline and after 21 d (12-h fast) and 22 d (36-h fast) of alternate-day fasting. Visual analogue scales were used to assess hunger weekly.

Results: Subjects lost 2.5 ± 0.5% of their initial body weight (P < 0.001) and 4 ± 1% of their initial fat mass (P < 0.001). Hunger increased on the first day of fasting and remained elevated (P < 0.001). RMR and RQ did not change significantly from baseline to day 21, but RQ decreased on day 22 (P < 0.001), which resulted in an average daily increase in fat oxidation of ≥15 g. Glucose and ghrelin did not change significantly from baseline with alternate-day fasting, whereas fasting insulin decreased 57 ± 4% (P < 0.001).

Conclusions: Alternate-day fasting was feasible in nonobese subjects, and fat oxidation increased. However, hunger on fasting days did not decrease, perhaps indicating the unlikelihood of continuing this diet for extended periods of time. Adding one small meal on a fasting day may make this approach to dietary restriction more acceptable. Am J Clin Nutr 2005;81:69–73.

KEY WORDS Resting metabolic rate, fat oxidation, insulin, glucose, biomarkers of longevity

INTRODUCTION

Prolonged dietary restriction (DR) is the only proven method of increasing the life span in rodents, flies, yeast, and worms (1). The mechanism or mechanisms by which DR increases life span are unclear, but the effects of DR include reduced metabolic rate, reduced oxidative damage, altered neuroendocrine signaling, and improved insulin sensitivity (2). The effect of prolonged DR on the life span in nonhuman primates is currently being investigated (3–5). Although conclusive results are years away, many improvements in biomarkers of longevity, including reduced core temperature, resting metabolic rate (RMR), dehydroepiandrosterone sulfate, glucose, and insulin, have already been observed. Prolonged DR also alters the expression of many genes from skeletal muscle, brain, and liver, including genes encoding heat shock proteins and uncoupling proteins and genes involved in oxidative damage (6–8). Recent microarray results in mouse liver indicate that there is significant overlap of genes that are up-regulated by short-term starvation and by prolonged DR (9).

Alternate-day fasting may therefore be an alternative to prolonged DR as a method of increasing maximal life span. Goodrick et al (10) found that alternate-day fasting increased median and maximal life span in C57Bl/6 mice when it was introduced at 1.5 and 6 mo of age and increased maximal, but not median, life span in A/J mice. Recently, Anson et al (11) observed that mice fed every other day consumed the same total energy as did ad libitum fed animals and had similar body weights but had reduced glucose and insulin concentrations and increased resistance to endotoxic stress (11).

A pilot study testing the feasibility and effects of long-term DR on biomarkers of longevity in nonobese humans is currently under investigation. This randomized clinical trial named CALERIE (sponsored by the National Institute of Aging) is testing numerous behavioral strategies and diets (ranging from liquid energy to 20–30% DR to increased energy expenditure by physical activity) to determine which of these will prove the most viable in today’s “obesogenic” environment. However, the feasibility and efficacy of alternate-day fasting is not being investigated. Given the difficulty that individuals have in estimating energy intake (12–14), alternate-day fasting may prove to be a less complicated method than prolonged DR in humans. Indeed, one study investigated the effects of alternate-day DR for 3 y (15). In that study, the subjects were allowed 1 L of milk and 2–3 pieces of fruit on their energy-restricted day and 9600 kJ/d on the other day. The control group was fed 9600 kJ/d every day. The subjects randomly assigned to alternate-day DR spent less time in the infirmary and had a lower death rate than in the control group (6 versus 13; NS) (16). The present study was undertaken to determine the feasibility of alternate-day fasting in nonobese subjects. In addition, the effects of alternate-day fasting on body weight, RMR, fat oxidation, and biomarkers of longevity were investigated.

1 From the Pennington Biomedical Research Center, Baton Rouge, LA.
2 Reprints not available. Address correspondence to E Ravussin, Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, LA, 70808. E-mail: ravusse@pbrc.edu.
Received May 27, 2004.
Accepted for publication September 2, 2004.
TABLE 1
Baseline characteristics of the participants by sex

<table>
<thead>
<tr>
<th></th>
<th>Men (n = 8)</th>
<th>Women (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>34 ± 3</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>80.6 ± 4.4</td>
<td>59.7 ± 1.7*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.2 ± 1.1</td>
<td>22.6 ± 0.6</td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td>22 ± 2</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.9 ± 0.4</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.0 ± 0.2</td>
<td>1.8 ± 0.1*</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/L)</td>
<td>2.5 ± 0.6</td>
<td>1.1 ± 0.1*</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>116 ± 2</td>
<td>104 ± 2*</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>75 ± 3</td>
<td>68 ± 2</td>
</tr>
</tbody>
</table>

1 All values are x ± SEM.
2 Significantly different from men, P < 0.01 (one-factor ANOVA).

SUBJECTS AND METHODS

Subjects

Healthy, nonobese [body mass index (in kg/m²) range: 20.0–30.0] men (n = 8) and women (n = 8) aged between 23 and 53 y were recruited (Table 1). The subjects had different levels of physical activity: 7 were sedentary, 3 were moderately active (exercised 1–2 times/wk), and 6 were quite active (exercised 4–5 times/wk). Competitive athletes and subjects with type 2 diabetes (exercised 1–2 times/wk), and 6 were quite active (exercised 4–5 times/wk). Competitive athletes and subjects with type 2 diabetes were excluded. The Institutional Review Board of the Pennington Biomedical Research Center approved the study, and the subjects gave their written informed consent.

Study design

The subjects attended the clinical research center on 2 consecutive days at baseline (days −2 and −1) and on 2 consecutive days after 3 wk of alternate-day fasting following a “feast” day (day 21) and following a “fast” day (day 22). The subjects had therefore fasted 12 h (overnight) on days −2, −1, and 21 and 36 h on day 22. The subjects were instructed to avoid exercise, alcohol, and coffee for ≥24 h before each visit. At each visit, the subjects arrived in the clinic at 0700 and were weighed while wearing a hospital gown. Blood pressure was measured with the subject in a seated position after a 5-min rest, oral temperature was recorded (SureTemp; Welch Allyn Inc, NY), and a fasting blood sample was drawn. RMR was measured for 30 min with a DeltaTrac metabolic monitor (SensorMedics, Yorba Linda, CA) after a 20-min resting period while the subjects were awake in a semirecumbent position. On days −2 and 21, body composition was measured by dual-energy X-ray absorptiometry (QDR 4500; Hologic Inc, Bedford, MA). At baseline and on days 1, 7, 15, and 21 (fasting days), the subjects completed visual analogue scales (VASs) at 1000, 1200, 1400, and 1600 to assess their feelings of hunger, fullness, desire to eat, satisfaction, and prospective food consumption (17). Briefly, the participants were asked to place a mark on a 100-mm line anchored by “not at all” and “extremely” to record subjective levels of hunger or satiety. The VASs were scored by measuring from the left end of the line to the mark in mm, and mean ratings were calculated for each day. At baseline, the subjects also completed the Eating Inventory questionnaire, which assessed dietary restraint (the intent and ability to restrict caloric intake), disinhibition (the tendency to overeat), and hunger (18). The subjects also completed a nine-item self-report questionnaire, which was developed for this study, to assess eating attitudes and behaviors with the use of an 8-point scale. This questionnaire (Eating Behaviors Questionnaire) assessed whether the subjects consider themselves “dieters” who watch what they eat or “big eaters” who tend to eat 1 or 2 large meals per day.

After baseline testing was completed, the subjects fasted from midnight to the subsequent midnight on alternating days for 22 d. On each fasting day, the subjects were allowed to consume energy-free beverages, tea, coffee, and sugar-free gum and were instructed to keep their water intake high. On each feeding day, the subjects were instructed to eat whatever they wished and were informed that double their usual food intake would be required to maintain their usual body weight. The subjects were provided with calibrated digital scales (Tanita, Arlington Heights, IL) to record their morning fasting body weight, urinary sticks to test for the presence of ketones, and a diet diary to record anything that was consumed on the fasting day. On day 20, the subjects were required to fast from 1900 so that a 12-h overnight fast would be completed before testing began the following morning at 0700. They did not break this fast until after their clinic visit on day 22.

Biochemical analytes

Glucose was analyzed by using a glucose oxidase electrode (Syncron CX7; Beckman, Brea, CA). Free fatty acids were measured on a Synchron CX5 by using reagents from Wako (Richmond, VA). β-Hydroxybutyrate was measured on a Synchron CX5 by using reagents from Sigma (St Louis). Insulin was measured by using an immunoassay on a DPC 2000 (Diagnostic Product Corporation, Los Angeles). Ghrelin was measured by using a radioimmunoassay kit from Linco (St Charles, MO).

Statistical analysis

Data are expressed as means ± SEMs. SAS 8.2 (SAS Institute Inc, Cary, NC) and SPSS 11.0.1 (SPSS Inc, Chicago) were used for data analysis. Baseline measures (days −2 and −1) were averaged. Statistics were performed by one- and two-factor repeated-measures analysis of variance. Post hoc analysis was performed with Tukey’s tests where necessary. RMR was analyzed by using linear regression to adjust for fat mass and fat-free mass. Correlations were performed with Pearson’s correlation coefficient. Significance was set at P < 0.05. Fasting insulin values below the detection limit of the assay (<2.0 mU/L) were assigned a value of 1.0 mU/L. Insulin values were log transformed for analysis.

RESULTS

The subjects’ characteristics by sex are given in Table 1. On the basis of their self-recorded diet diaries and weight logs (Figure 1), the subjects complied with the protocol. Urinary ketones were not useful as a measure of compliance because they were not consistently detected in all subjects (data not shown). On the basis of daily regressed body weights, the subjects lost 2.5 ± 0.5% of their initial body weight. This self-reported weight loss was confirmed by weights measured in the clinic at baseline and on days 21 and 22 (P < 0.001). Significant reductions were observed in fat mass (P < 0.001) and fat-free mass (P < 0.05) after the intervention (Figure 1).
On average, the men considered themselves “big eaters,” and the women reported that they “watched what they ate.” Percentage weight loss did not differ significantly between the men and the women, but weight loss correlated negatively with considering oneself a big eater after adjustment for sex ($r = -0.63, P = 0.04$). The dietary restraint and disinhibition scales of the Eating Inventory questionnaire did not significantly predict weight loss.

VASs were completed for all days by only 8 of 16 subjects. First, baseline results were compared with the first day of fasting. As expected, a significant increase was found in feelings of hunger (from $37 \pm 5$ to $56 \pm 4$ mm; $P < 0.001$), and a significant decrease was noted in feelings of fullness (from $43 \pm 3$ to $23 \pm 4$ mm; $P < 0.001$). However, repeated-measures analysis over time (days 1, 7, 15, and 21) showed no significant changes in the subjects’ perception of hunger, thirst, desire to eat, or feelings of satisfaction, although feelings of fullness increased slightly over time ($P < 0.05$).

Temperature (data not shown) and absolute and relative resting metabolic rate (adjusted for fat-free mass and fat mass) were not significantly different from baseline (Table 2). Respiratory quotient (RQ) was also not significantly different from baseline at day 21; however, RQ was lower on day 22 ($P < 0.001$; Table 2). More specifically, fat oxidation increased from 64 g/24 h at baseline to 101 g/24 h, and carbohydrate oxidation decreased from 175 to 81 g/24 h. The change in RQ from baseline to day 21 was related to weight loss ($r = -0.76, P < 0.001$).

The women had significantly lower glucose, insulin, free fatty acid, triacylglycerol, and LDL-cholesterol concentrations and significantly higher HDL-cholesterol and ghrelin concentrations than did the men ($P < 0.05$). Fasting glucose was not significantly changed from baseline in the men or the women (Figure 2). Fasting insulin was lower on day 22 in both the men and the women ($P < 0.001$), and fasting β-hydroxybutyrate and free fatty acid concentrations were higher on day 22 in both the men and the women (Figure 2; $P < 0.001$). Fasting ghrelin was not significantly altered from baseline on day 21 (results not shown) or day 22 (from 1019 ± 128 to 1063 ± 158 pg/mL in the men and from 1403 ± 63 to 1493 ± 139 pg/mL in the women). Systolic and diastolic blood pressure were not significantly altered by the intervention (data not shown). HDL was elevated from baseline in the women only ($P < 0.001$; data not shown), and triacylglycerol was significantly reduced from baseline in the men only ($P < 0.05$; data not shown).

**DISCUSSION**

Alternate-day fasting may be an alternative to prolonged DR for increasing the life span (11). In the present study, we report that alternate-day fasting is feasible for short time periods in nonobese subjects. One participant reported feeling light-headed once, and 4 subjects reported constipation. No subjects withdrew during the study, but many reported feeling irritable on their fasting days, perhaps indicating the unlikelihood of continuing this diet for extended periods of time. The results from the VASs suggest that feelings of fullness may have increased from the first fasting day over the course of the study, but other subjective states related to food intake motivation did not habituate, including hunger. This result contrasts with the results of studies using liquid-based, very-low-energy diets where hunger diminishes despite a marked energy deficit (19). Overall, these results suggest that a prolonged schedule of fasting and feasting would be marred by aversive subjective states (eg, hunger and irritability), which would likely limit the ability of most individuals to sustain this eating pattern.

This is the first study, to our knowledge, to test the effects of alternate-day fasting on body weight and other metabolic variables in humans. Body weight was clearly reduced from baseline after 3 wk of alternate-day fasting, indicating that the subjects were unable to consume enough food on the fasting days to

**TABLE 2**

Resting metabolic rate (RMR), respiratory quotient (RQ), and fat and carbohydrate oxidation measured at baseline and after a fed day (day 21) and a fast day (day 22)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Day 21</th>
<th>Day 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMR (kJ/d)</td>
<td>6675 ± 283</td>
<td>6292 ± 268</td>
<td>6329 ± 260</td>
</tr>
<tr>
<td>RQ</td>
<td>0.85 ± 0.01</td>
<td>0.86 ± 0.02</td>
<td>0.79 ± 0.01</td>
</tr>
<tr>
<td>Fat oxidation (g/24 h)</td>
<td>64 ± 8</td>
<td>54 ± 10</td>
<td>101 ± 9*</td>
</tr>
<tr>
<td>Carbohydrate oxidation (g/24 h)</td>
<td>175 ± 17</td>
<td>184 ± 24</td>
<td>81 ± 16*</td>
</tr>
</tbody>
</table>

*Significantly different from baseline, $P < 0.001$ (one-factor repeated-measures ANOVA).

All values are $\bar{x} \pm$ SEM. Two consecutive days at baseline were averaged for analysis.

Calculated by assuming that protein oxidation was 15% of RMR.
maintain their weight. This is opposite the results observed in rodents, where mice fed every other day maintained their body weight and consumed roughly the same amount of food in 1 d that ad libitum–fed animals consumed over 2 d (11). We hypothesized that the subjects with a self-reported ability to overeat or eat large amounts of food would maintain their body weight, and this hypothesis was supported: considering oneself a “big eater” was negatively associated with weight loss when sex was controlled for by partial correlation. Whether alternate-day fasting would lead to weight loss in obese participants remains unclear. The negative subjective states associated with the study cast doubt on the ability of individuals to voluntarily engage in alternate-day fasting for prolonged periods of time. Altering the clock time that the subjects are asked to fast (eg, from 1900 to 1900) or adding a small meal (10–20% of caloric needs) to the fasting day may make alternate-day fasting more acceptable in all populations.

Ghrelin is a peptide secreted in the gut that is reduced on feeding and has been implicated in the regulation of feeding behavior and energy balance. Obese subjects have lower fasting ghrelin concentrations than do lean subjects (20) but have impaired suppression of plasma ghrelin in response to a meal (21). Furthermore, ghrelin is increased after weight loss in obese subjects (22, 23), perhaps driving the common phenomenon of weight regain after weight loss. In the present study, the women had significantly higher ghrelin concentrations than did the men. This has been reported previously (24) but is not consistently observed (25) and may be related to central adiposity. In contrast with the large increases in reported hunger, plasma ghrelin was unchanged in both the men and the women, even after 36 h of fasting. Studies in rodents have found that 24-h fasts increase plasma ghrelin (26). However, fasting for 72 h did not change plasma ghrelin in lean men (24). The results of these fasting studies in humans call into question the role of ghrelin in the hunger drive and highlight the need for further research in this area.

A hallmark of rodent studies of longevity is reduced fasting glucose and insulin concentrations and increased insulin sensitivity in dietary-restricted animals (27). Reduced fasting insulin has also been associated with increased longevity in humans (27). In the present study, insulin was reduced after a fast day, suggesting improved insulin sensitivity. However, plasma free fatty acids were also elevated after fasting; these elevated concentrations may impair insulin-mediated glucose disposal and the suppression of hepatic glucose production (28). We also found that alternate-day fasting did not significantly change fasting glucose or insulin from baseline after a 12-h fast. This is in contrast with results in mice, in which glucose and insulin concentrations were lower after 14-h fasts than in ad libitum fed–mice or mice fed energy-restricted diets. Thus, humans may need to fast for longer than 12 h for this effect to be observed. Alternatively, this could be due to the already low glucose concentrations of our population or that 3 wk of alternate-day fasting was insufficient to produce this response. The study design may also have affected these results, because the subjects anecdotally reported eating even more than usual on day 20 (knowing they were about to enter a longer than usual fast day).

RMR was not significantly changed after 3 wk of alternate-day fasting. The effects of 36-h fasts on RMR have not been previously reported. Horton and Hill (29) observed no differences in metabolic rate (measured for 12 h in a metabolic chamber after a mixed meal) between overnight or 3-d fasts. We did observe that subjects oxidized more fat on day 22 as evidenced by a reduction in RQ from 0.85 to 0.79. However, RQ was not altered on day 21. This suggests that there were no sustained increases in fat oxidation on fed days. Caution must be exercised when interpreting this result, because the subjects did not consume standardized

![FIGURE 2. Mean (±SEM) fasting glucose, fasting insulin, fasting β-hydroxybutyrate (BHBA), and fasting free fatty acids (FFA) at baseline, day 21, and day 22 in men (n = 8) and women (n = 8). *Significantly different from baseline, P < 0.01 [two-factor (time and sex) repeated-measures ANOVA].](image-url)
diets and RQ is heavily dependent on fat intake and energy balance. However, it is more likely that we underestimated fat oxidation, because the subjects were coming out of positive energy balance and because overall fat oxidation was increased by an average of $\geq 15$ g/d. Furthermore, because weight loss is positively correlated with increased fat oxidation, the results suggest that the subjects with a greater ability to oxidize fat lost more weight. Alternatively, it could be argued that the subjects who had a greater caloric deficit had increased fat oxidation.

In conclusion, alternate-day fasting is feasible in nonobese subjects for short time periods, although unlike rodents, the subjects were unable to maintain their body weight. Furthermore, fat oxidation was increased and translated into fat mass loss. Hunger on fasting days did not habituate over the course of the study, which perhaps indicates the unlikelihood of subjects continuing on this diet for extended periods of time. Whether alternate-day fasting would promote weight loss in an obese population is uncertain.

We acknowledge the clinical research staff for their assistance in performing this study and Julia Volaufova for assistance with the statistical analysis. LKH, SRS, and ER were involved in developing the study protocol and the experimental design. CKM and SA administered and analyzed the VAS and psychological questionnaires. LKH wrote the draft manuscript with contributions from ER, SRS, and CKM. None of the authors had any financial interests in organizations sponsoring this research.

**REFERENCES**


Comparison of multifrequency bioelectrical impedance analysis with dual-energy X-ray absorptiometry for assessment of percentage body fat in a large, healthy population

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ABSTRACT

Background: Bioelectrical impedance analysis (BIA) is widely used in clinics and research to measure body composition. However, the results of BIA validation with reference methods are contradictory, and few data are available on the influence of adiposity on the measurement of body composition by BIA.

Objective: The goal was to determine the effects of sex and adiposity on the difference in percentage body fat (%BF) predicted by BIA compared with dual-energy X-ray absorptiometry (DXA).

Design: A total of 591 healthy subjects were recruited in Newfoundland and Labrador. %BF was predicted by using BIA and was compared with that measured by DXA. Methods agreement was assessed by Pearson’s correlation and Bland and Altman analysis. Differences in %BF among groups based on sex and adiposity were analyzed by using one-factor analysis of variance with Bonferroni correction.

Results: Correlations between BIA and DXA were 0.88 for the whole population, 0.78 for men, and 0.85 for women. The mean %BF determined by BIA (32.89 ± 8.00%) was significantly lower than that measured by DXA (34.72 ± 8.66%). The cutoffs were sex specific. BIA overestimated %BF by 3.03% and 4.40% when %BF was <15% in men and <25% in women, respectively, and underestimated %BF by 4.32% and 2.71% when %BF was >25% in men and >33% in women, respectively.

Conclusions: BIA is a good alternative for estimating %BF when subjects are within a normal body fat range. BIA tends to overestimate %BF in lean subjects and underestimate %BF in obese subjects.

KEY WORDS Percentage body fat, multifrequency bioelectrical impedance analysis, dual-energy X-ray absorptiometry, Newfoundland population, body composition

INTRODUCTION

Body-composition information, including percentage body fat (%BF), is widely used to evaluate growth and nutrition in children (1, 2) and nutritional status in various disease conditions, such as AIDS (3), gastrointestinal disease (4), Crohn disease (5), and renal and various other diseases (6, 7). Body-composition assessment has many clinical uses, such as assessing disease progression or treatment efficacy (3–7). Body-composition measurements are also used to assess athletes (8, 9) and can be used in the study of aging (10).

Many technologies are available to measure body composition, such as the underwater weighing method, which has been used as a traditional standard (11). Air-displacement plethysmography and dual-energy X-ray absorptiometry (DXA) are 2 relatively new reference methods (12–14). The use of these methods is limited, however, because of inaccessibility and the high cost of equipment. Thus, simple methods such as bioelectrical impedance analysis (BIA) and skinfold-thickness measurements are still the norm in field studies and for public use (15–17).

The BIA method has been widely used in clinics, in sports medicine, and in weight reduction programs (18, 19). Several studies have compared predictions of %BF by BIA with measurements made by reference methods (20–24), but the results are contradictory. Some studies showed that %BF is overestimated by BIA (25, 26), whereas others suggested that BIA underestimates %BF (27–29). Some studies showed good agreement between BIA and DXA (21, 30, 31), whereas others indicated that the BIA method lacks precision and accuracy (20, 32, 33). It seems that the error of the BIA method in comparison with reference methods is greater in patients with chronic renal failure than in healthy subjects (34). In addition, the degree of adiposity appears to influence the variation in %BF measured by BIA in children (35, 36). Questions such as whether BIA tends to over- or underestimate %BF when compared with DXA and the extent of this bias remain unanswered because most of the studies were performed with small sample sizes and in patients with different diseases.

To answer the above questions, we performed parallel measurements of %BF by using both multifrequency BIA and DXA in a large sample of the healthy population in Newfoundland and

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2 Supported in part by the Canadian Foundation for Innovation and the Canadian Institute for Health Research. GS is chair of pediatric genetics, sponsored by Novartis Pharmaceuticals.
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Labor, DXA is arguably one of the best reference methods to use: it has been validated against other methods of body composition analysis and has shown little bias based on age, sex, physical activity levels, race, or proportion of fat (14).

SUBJECTS AND METHODS

Subjects

All 591 subjects were recruited from the Canadian province of Newfoundland and Labrador (NL) for a large-scale genetic study of human complex diseases. Subjects were self-selected through a poster campaign advertising the project. Responders gave their written consent and then completed a screening questionnaire that included basic personal information, such as physical characteristics and health status. Persons who met the following criteria were eligible to participate in the study: age between 19 and 60 y, born in NL and family has lived in NL for 3 generations, that included basic personal information, such as physical characteristics and health status. Persons who met the following criteria were eligible to participate in the study: age between 19 and 60 y, born in NL and family has lived in NL for 3 generations, and healthy without serious metabolic, cardiovascular, or endocrine disease. The study was approved by the Human Investigation Committee of the Faculty of Medicine, Memorial University of Newfoundland.

Measurements of percentage body fat

All measurements were performed after the subjects had fasted for 12 h. The subjects were weighed in standardized light clothes and without shoes on a platform manual scale balance (Health O Meter Inc, Bridgeview, IL).

DXA (Lunar Prodigy; GE Medical Systems, Madison, WI) was used for the measurement of whole-body composition, including fat mass, lean body mass (comprising muscle, internal organs, and bone water), and bone mineral densities. %BF was calculated from entire body mass (including bone mineral densities) by using the manufacturer’s software (version 4.0). The subjects had undergone no nuclear examination within the previous 4 wk, and the female subjects were not pregnant at the time of examination. All metal items were removed from the volunteer to ensure accuracy of the measurement. DXA measurements were performed while the subject was lying in a supine position. BIA measurements were done immediately after the DXA analysis.

BIA measurements were carried out with the subject lying in a supine position on a flat, nonconductive bed by using a multifrequency tetrapolar technique (QuadScan 4000; Bodystat, Douglas, United Kingdom). The Bodystat QuadScan 4000 unit has 4 electrodes. Two electrodes were placed on the right wrist with one just proximal to the third metacarpophalangeal joint (positive) and one on the wrist next to the ulnar head (negative). Two electrodes were placed on the right ankle with one just proximal to the third metatarsophalangeal joint (positive) and one between the medial and lateral malleoli (negative). Multifrequency (5, 50, 100, and 200 kHz) currents were introduced from the positive leads and traveled throughout the body to the negative leads. %BF was calculated by using the manufacturer’s software.

Statistical analysis

All data are reported as means ± SDs. Paired t tests were used to compare %BF measured by BIA and DXA. The correlation between %BF predicted by BIA and that measured by DXA was estimated by the use of Pearson’s correlation. %BF values < 0.05 were considered significant. Bias was calculated as the mean of the difference ± 1.96 SDs by using the Bland and Altman analysis (37). Differences in %BF between the BIA and DXA methods among groups of lean and obese subjects (according to DXA) were tested by using one-factor analysis of variance (ANOVA) and were corrected based on the Bonferroni method. Multivariate regression analysis was performed to determine the influence of possible confounding factors on %BF predicted by BIA among groups based on adiposity. Cutoffs for lean and obese groups according to %BF in men and women were adapted from Bray (38). SPSS for WINDOWS (version 11.5; SPSS Inc, Chicago) was used to perform the statistical analysis.

RESULTS

Physical characteristics of the subjects

The subjects’ basic physical characteristics are summarized in Table 1. A total of 591 eligible volunteers (491 women and 100 men) participated in the study. The subjects’ ages ranged from 19

### TABLE 1

Physical characteristics of the subjects

<table>
<thead>
<tr>
<th>Description</th>
<th>All subjects (n = 591)</th>
<th>Men (n = 100)</th>
<th>Women (n = 491)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>42.15 ± 10.27 (19, 60)</td>
<td>39.65 ± 12.5 (19, 60)</td>
<td>42.66 ± 9.70 (19, 60)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>70.99 ± 14.68 (45, 157)</td>
<td>84.24 ± 13.53 (55, 137)</td>
<td>68.31 ± 13.41 (45, 157)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164.24 ± 7.7 (135, 198)</td>
<td>174.99 ± 7.49 (157, 198)</td>
<td>162.07 ± 5.6 (135, 178)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.28 ± 4.94 (16.9, 54.27)</td>
<td>27.53 ± 4.39 (19.87, 43.53)</td>
<td>26.03 ± 5.0 (16.98, 55.27)</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.89 ± 0.07 (0.73, 1.24)</td>
<td>0.96 ± 0.07 (0.74, 1.20)</td>
<td>0.87 ± 0.07 (0.73, 1.24)</td>
</tr>
</tbody>
</table>

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1. All values are x ± SD; minimum and maximum in parentheses.
2. *Significantly different from DXA, P < 0.001 (paired t test).

### TABLE 2

Percentage body fat measured by bioelectrical impedance analysis (BIA) and by dual-energy X-ray absorptiometry (DXA)

<table>
<thead>
<tr>
<th>Method</th>
<th>All subjects (n = 591)</th>
<th>Men (n = 100)</th>
<th>Women (n = 491)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIA</td>
<td>32.89 ± 8.00 (10.6, 58.3)</td>
<td>22.67 ± 6.12 (10.6, 37.7)</td>
<td>34.93 ± 6.73 (17.0, 58.3)</td>
</tr>
<tr>
<td>DXA</td>
<td>34.72 ± 8.66 (4.6, 59.9)</td>
<td>25.03 ± 7.17 (7.4, 41.6)</td>
<td>36.68 ± 7.55 (4.6, 59.9)</td>
</tr>
</tbody>
</table>

---

1. All values are x ± SD; minimum and maximum in parentheses.
2. *Significantly different from DXA, P < 0.001 (paired t test).
to 60 y, and the men were 3.0 y younger than the women on average. The men were also 15.93 kg heavier and 12.9 cm taller than the women on average. The subjects had a wide range of body mass indexes (BMIs; in kg/m²): from 16.98 to 55.27. Waist-to-hip ratio was larger in the men.

Comparison of %BF measured by BIA and DXA

The general correlation coefficient ($r$) between the 2 methods for men and women combined was 0.88. The correlation coefficients between BIA and DXA for men and women were 0.78 and 0.85, respectively.

The results of %BF measured by both BIA and DXA are shown in Table 2. The mean %BF obtained by BIA in all subjects was significantly lower than that measured by DXA: 32.89 ± 8.00% compared with 34.72 ± 8.66%. The results were similar when the analysis was performed according to sex.

BIA bias on the basis of %BF

When the subjects were stratified according to their %BF (low, moderate, or high), significant differences in the BIA-DXA comparisons were apparent. The agreement between BIA and DXA was almost perfect when the subjects’ %BF was not low or high. As shown in Figure 1, BIA actually overestimated %BF by 3.56% for subjects whose %BF was <20% (lean). BIA underestimated %BF by 2.65% for subjects whose %BF was >30% (obese), and the 2 methods fit each other well when %BF was between 20% and 30% (normal). The cutoffs for lean and obese categories were sex specific. As shown in Figure 2 for men, BIA overestimated %BF by 3.03% when %BF was <25% (lean), underestimated %BF by 4.32% when %BF was >25% (obese), and had little difference with DXA when %BF fell between 25% and 25% (normal). As shown in Figure 3 for women, BIA overestimated %BF by 4.40% when %BF was <25% (lean), underestimated %BF by 2.71% when %BF was >33% (obese).

The agreement in %BF between BIA and DXA was compared by using the Bland and Altman analysis in all subjects together and in men and women separately. The mean differences in %BF between the BIA and DXA methods were $-1.83 ± 4.10$% for all subjects, $-2.16 ± 4.56$% for men, and $-1.77 ± 4.00$% for women.

### TABLE 3

Multivariate linear regression analysis of the difference between measurements of percentage body fat by bioelectrical impedance analysis and dual-energy X-ray absorptiometry (DXA) according to adiposity and independent variables

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>$\beta$ ± SE</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXA</td>
<td>$-0.663 ± 0.019$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI</td>
<td>$0.639 ± 0.030$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age</td>
<td>$0.209 ± 0.009$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex</td>
<td>$8.542 ± 0.370$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>$1.434 ± 1.543$</td>
<td>0.353</td>
</tr>
</tbody>
</table>

$^1$ Model $r^2 = 0.73$, $n = 591$. $\beta$ = unstandardized coefficient. For sex, male = 0, female = 1.
and had little difference with DXA when %BF fell between 25% and 33% (normal). All of these differences between groups were significant by one-factor ANOVA, except for the comparison between lean and normal-weight men. The results were corrected according to the Bonferroni method.

Multivariate regression analysis was performed to explore the role of physical variables on the adiposity-dependent bias of BIA when compared with DXA. Although variables such as BMI, age, and sex significantly contributed to the variation in %BF predicted by BIA, the influence of adiposity remained significant after adjustment for these factors (Table 3).

DISCUSSION

Body-composition information is extensively used in clinics, sports medicine, and other health-related fields (1–8). Reference methods such as DXA, air-displacement plethysmography, and underwater weighing can provide accurate results; however, these methods are costly and often inaccessible to the public (11–14). In most situations, BIA and other field methods are the only techniques available for body-composition measurements.

The results of the present study bridge the gap between previous contradictory studies and provide reliable information on the correct interpretation of %BF analysis by BIA. Our study design featured 2 aspects that improved on previous investigations. First, our sample size of 591 persons is larger than any other BIA validation study to our knowledge. Second, we covered a wider range of %BF than in previous studies, which is more representative of a healthy population. In addition, our female cohort, which comprised 491 women, is much larger than the sample size used to develop the QuadScan prediction algorithm and covers a wider range of %BF (IJ Meeuwsen, Bodystat Ltd, personal communication, 2004). Thus, we identified significant bias caused by adiposity that was not detected and considered in the development of the QuadScan 4000 prediction equation.

The correlations between BIA and DXA for %BF were generally good, which is consistent with other reports (18–28, 30, 31). The agreement between BIA and DXA was assessed by use of the Bland and Altman analysis in all subjects combined and in men and women separately. The correlation coefficient was highest when all subjects were analyzed together and was reduced when the subjects were stratified by sex. However, the correlation coefficient alone is not sufficient to prove the equivalence of the 2 methods (37). When the mean value of %BF was compared between the 2 methods, BIA always underestimated %BF in the combined subjects and in men and women separately. Although the absolute values of the mean difference between the 2 methods were small, the agreement may not be suitable for measurement of body composition in individual subjects. The Bland and Altman analysis shows that the individual variations are large, with the variation being greater in men than in women (−2.26 ± 4.62% compared with −1.80 ± 4.05%).

The major contribution of the present study is the demonstration that the size and direction of the BIA-DXA difference in adults is dependent on the %BF of the subject (low, medium, or high). BIA tends to overestimate body fat when subjects are relatively lean and underestimate body fat when subjects are overweight or obese. Furthermore, the cutoffs between these groups differ in men and women. BIA tends to overestimate body fat when %BF is <15% in men and <25% in women (lean). BIA tends to underestimate body fat when %BF is >25% in men and >33% in women (obese). These cutoffs define obesity according to %BF (38). Little difference was seen when BIA was used in men with %BF between 15% and 25% and in women with %BF between 25% and 33% (normal). These findings have important implications for the use of BIA measurements and the interpretation of results.

Multivariate linear regression analysis was performed to explore the role of physical variables on the variation in %BF measured by BIA among groups based on adiposity. Our model shows that the variation in the %BF measurements by the 2 methods can be explained by adiposity, as determined by DXA. Variables such as BMI, age, and sex significantly improved the amount of explained variation in our model; however, the influence of adiposity remained significant after adjustment for these factors. We therefore conclude that the variation in predicted %BF by BIA among adiposity groups could be reduced by adjusting for adiposity, BMI, age, and sex.

In summary, parallel measurement of %BF by BIA and DXA showed that BIA analysis must be carefully interpreted when used on lean, overweight, or obese persons. BIA tends to underestimate body fat in all subjects and in men and women separately. This bias, however, depends on the degree of adiposity. In lean subjects, BIA tends to overestimate %BF. In overweight or obese subjects, BIA tends to underestimate %BF. Because BIA is used to measure body composition in a variety of clinical settings, such as in patients with AIDS wasting (3) or chronic obesity (39), this bias must be taken into consideration when interpreting BIA data.

We thank the volunteers who participated in the present study and the following persons for their contributions to volunteer recruitment and data input: Yang Zhang, Christiane Dawe, Dax Rumsey, James Thorburn, Amber Snow, Aihua Ma, Sandra Cooke, Elizabeth Drover, and Aman Gill.

GS was responsible for study design, data analysis, and writing of the manuscript. CRF was responsible for data collection, statistical analysis, and revision of the manuscript. GRM, HZ, WG, JRB, DGF, BY, RCG, and Y-GX were responsible for data collection. MM was involved in statistical analysis. GS holds the position of chair of pediatric genetics, which is supported by Novartis Pharmaceuticals. None of the other authors had any conflicts of interest to disclose.

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33. Tsai AC, Sandretto A, Chung YC. Dieting is more effective in reducing weight but exercise is more effective in reducing fat during the early phase of a weight-reducing program in healthy humans. J Nutr Biochem 2003;14:541–9.


Evaluation of handgrip strength as a nutritional marker and prognostic indicator in peritoneal dialysis patients

Angela Yee-Moon Wang, Mandy Man-Mei Sea, Zoe So-Ying Ho, Siu-Fai Lui, Philip Kam-Tao Li, and Jean Woo

ABSTRACT

Background: Serum albumin has limitations as a nutritional marker in patients undergoing peritoneal dialysis (PD) in that it is affected by inflammation, systemic disease, overhydration, and urinary and dialysate protein loss. Handgrip strength is a simple, easily performed bedside test that has been shown to correlate with lean body mass in patients close to inception of dialysis.

Objective: We evaluated the associations of handgrip strength with other clinical factors and examined its relations with mortality and cardiovascular death in PD patients.

Design: We prospectively enrolled 233 chronic PD patients and assessed handgrip strength and other variables at baseline and then followed the patients for a mean (±SD) of 30 ± 14 mo.

Results: Baseline handgrip strength was significantly associated with age, sex, height, diabetes, residual glomerular filtration rate (GFR), and hemoglobin but not with C-reactive protein (CRP). After adjustment for age, sex, and height, handgrip strength was most strongly correlated with lean body mass on the basis of creatinine kinetics (r = 0.334, P < 0.001), followed by serum albumin and subjective global assessment. Both men and women who died had lower handgrip strengths than did those who remained alive (P < 0.001). After control for age, sex, diabetes, atherosclerotic vascular disease, GFR, hemoglobin, CRP, and serum albumin, greater handgrip strength was predictive of lower all-cause [hazards ratio (HR): 0.95 (95% CI: 0.92, 0.99); P = 0.005] and cardiovascular [HR: 0.94 (0.90, 0.98); P = 0.004] mortality.

Conclusions: Handgrip strength not only is a marker of body lean muscle mass but also provides important prognostic information independent of other covariates, including CRP and serum albumin. Our data suggest that handgrip strength may be used in conjunction with serum albumin as a nutrition-monitoring tool in patients undergoing PD.

KEY WORDS
Handgrip strength, nutrition, outcome, peritoneal dialysis

INTRODUCTION

Protein-energy malnutrition is a frequent complication in patients with end-stage renal disease (1–3) and is well known to be associated with cardiac comorbidity and inflammation (4) and predictive of poor survival in dialysis patients (5, 6). Several different variables have been used to assess nutritional status: serum albumin, subjective global assessment (SGA), dietary protein intake, handgrip strength (HGS), and measurements of lean body mass (LBM) made by using anthropometry, creatinine kinetics (CK), or dual-energy X-ray absorptiometry. Of these, serum albumin is the most frequently used. Indeed, several studies, including the CANUSA study (5), have shown the importance of serum albumin in predicting morbidity and mortality in dialysis patients. However, there is increasing evidence that serum albumin has its limitations as a nutritional marker. The presence of a systemic inflammatory response is an important contributing factor to hypoalbuminemia (7, 8). Peritoneal albumin loss is also partly responsible for hypoalbuminemia in peritoneal dialysis (PD) patients (9). Other factors, such as systemic diseases and old age, also affect serum albumin in dialysis patients (9). Furthermore, serum albumin may change with the hydration status of patients, and hypoalbuminemia may be the result of not only malnutrition but also plasma volume expansion (8, 10). Hence, it becomes imperative to identify a marker that assesses nutritional status more reliably than does serum albumin and better predicts outcome in dialysis patients.

HGS is a simple, quick, easily performed, inexpensive, and readily available bedside test. A previous study reported that HGS in end-stage renal disease patients close to inception of dialysis shows a strong positive correlation with LBM (11), which suggests that HGS may be a direct marker of body lean muscle mass. Indeed, HGS has been used as a nutritional marker in hemodialysis patients (1). Studies in other population groups also showed a similar association between a low HGS and poor nutritional status (12, 13). Interestingly, one study showed that HGS is an independent predictor of outcome in male patients with end-stage renal disease who are close to the inception of dialysis therapy (14). However, that study included both patients who were subsequently treated with hemodialysis and those who were treated with PD. Because the factors that predict outcome in hemodialysis patients may not necessarily apply to PD patients, it is imperative to evaluate whether HGS is a useful nutritional marker and has prognostic implication in the PD population. Forearm muscle strength was measured as opposed to the strength of a larger muscle associated with standing and walking.
Subjects and Methods

We prospectively enrolled 233 patients (120 men and 113 women) who had been receiving continuous ambulatory PD treatment for >3 mo. All patients were dialyzed by using conventional glucose-based, lactate-buffered PD solutions. Exclusion criteria included having an underlying malignancy; ongoing active inflammatory disease, such as systemic lupus erythematosus or rheumatoid arthritis; tuberculous infection and still receiving treatment; and chronic obstructive airway disease. The study protocol was approved by the Human Research Ethics Committee of the Chinese University of Hong Kong. Informed consent was obtained from all patients at the time of study enrollment. In the event of an acute medical problem, such as pneumonia, peritonitis, or exit site infection, the assessment of nutritional status, inflammation, and indexes of dialysis adequacy were deferred for ≥1 mo after complete resolution of the acute event.

Nutritional status was assessed by using SGA, body mass index, serum albumin, HGS, and LBM as estimated by CK. SGA was performed by experienced research staff blinded to all clinical and biochemical variables of the patients. The SGA includes 6 subjective assessments: 3 based on the patient’s history of weight loss, presence of anorexia, and vomiting and 3 based on the physician’s grading of muscle wasting, presence of edema, and loss of subcutaneous fat (3, 16). Edema is not a useful index of malnutrition (16), but its presence or absence must be taken into account when assessing changes in body weight. On the basis of these assessments, each patient’s nutritional status was scored as follows: 1 = normal nutrition, 2 = mild malnutrition, and 3 = moderate and severe malnutrition. Body weight was measured by using a scale after the patient’s abdomen had been drained of peritoneal fluid.

HGS was measured by using the Smedley handy dynamometer (Sportstek, Victoria, Australia) by experienced research staff blinded to all clinical and biochemical data of the patients. The SGA includes 6 subjective assessments: 3 based on the patient’s history of weight loss, presence of anorexia, and vomiting and 3 based on the physician’s grading of muscle wasting, presence of edema, and loss of subcutaneous fat (3, 16). Edema is not a useful index of malnutrition (16), but its presence or absence must be taken into account when assessing changes in body weight. On the basis of these assessments, each patient’s nutritional status was scored as follows: 1 = normal nutrition, 2 = mild malnutrition, and 3 = moderate and severe malnutrition. Body weight was measured by using a scale after the patient’s abdomen had been drained of peritoneal fluid.

The patients were asked to bring 24-h urine and dialysate samples on the morning of the nutritional assessment for measurement of urea and creatinine concentrations. LBM was calculated by using CK, as suggested by Keshaviah et al (17).

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**Table 1**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (n = 233)</th>
<th>Men (n = 120)</th>
<th>Women (n = 113)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>55 ± 12⁴</td>
<td>57 ± 12</td>
<td>53 ± 12</td>
<td>0.015</td>
</tr>
<tr>
<td>Duration of dialysis (mo)</td>
<td>26 (14, 49)⁴</td>
<td>25 (14, 44)</td>
<td>28 (14, 62)</td>
<td>0.053</td>
</tr>
<tr>
<td>History of smoking (%)</td>
<td>38</td>
<td>63</td>
<td>11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>30</td>
<td>31</td>
<td>28</td>
<td>0.674</td>
</tr>
<tr>
<td>Atherosclerotic vascular disease (%)</td>
<td>22</td>
<td>26</td>
<td>19</td>
<td>0.184</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>58 ± 10</td>
<td>62 ± 10</td>
<td>54 ± 9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body height (m)</td>
<td>1.58 ± 0.08</td>
<td>1.63 ± 0.06</td>
<td>1.53 ± 0.06</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.2 ± 3.4</td>
<td>23.1 ± 3.1</td>
<td>23.2 ± 3.7</td>
<td>0.782</td>
</tr>
<tr>
<td>Subjective global assessment (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well nourished</td>
<td>56</td>
<td>54</td>
<td>58</td>
<td>0.669</td>
</tr>
<tr>
<td>Mildly malnourished</td>
<td>29</td>
<td>28</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Moderately or severely malnourished</td>
<td>16</td>
<td>18</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>LBMI by CK (kg)</td>
<td>32.0 ± 7.3</td>
<td>35.3 ± 7.5</td>
<td>28.4 ± 5.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>9.2 ± 1.7</td>
<td>9.5 ± 1.8</td>
<td>8.9 ± 1.5</td>
<td>0.003</td>
</tr>
<tr>
<td>Serum albumin (g/L)</td>
<td>28.7 ± 5.2</td>
<td>28.7 ± 5.0</td>
<td>28.7 ± 5.3</td>
<td>0.907</td>
</tr>
<tr>
<td>Handgrip strength (kg)</td>
<td>19.9 ± 10.6</td>
<td>24.8 ± 10.3</td>
<td>14.7 ± 8.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total weekly Kt/V</td>
<td>1.81 ± 0.44</td>
<td>1.68 ± 0.42</td>
<td>1.95 ± 0.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Peritoneal dialysis Kt/V</td>
<td>1.51 ± 0.37</td>
<td>1.35 ± 0.32</td>
<td>1.68 ± 0.34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total weekly CCr (L · wk⁻¹ · 1.73 m⁻²)</td>
<td>57 ± 21</td>
<td>58 ± 22</td>
<td>55 ± 20</td>
<td>0.420</td>
</tr>
<tr>
<td>Residual GFR (mL · min⁻¹ · 1.73 m⁻²)</td>
<td>0.64 (0.195)</td>
<td>0.87 (0.237)</td>
<td>0.37 (0.163)</td>
<td>0.012</td>
</tr>
<tr>
<td>nPNA (g · kg⁻¹ · d⁻¹)</td>
<td>0.96 ± 0.19</td>
<td>0.97 ± 0.19</td>
<td>0.95 ± 0.20</td>
<td>0.570</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>2.60 (0.91, 7.87)</td>
<td>2.19 (0.89, 6.37)</td>
<td>3.00 (0.92, 9.00)</td>
<td>0.686</td>
</tr>
</tbody>
</table>

¹ LBM, lean body mass; CK, creatinine kinetics; Kt/V, urea clearance; CCr, creatinine clearance; GFR, glomerular filtration rate; nPNA, normalized protein-equivalent nitrogen appearance.
² P values for comparison between men and women by t test for data expressed as x ± SD, by Mann-Whitney test for data expressed as median (interquartile range), or by chi-square test for categorical data.
³ x ± SD (all such values).
⁴ Median; interquartile range in parentheses (all such values).
The adequacy of dialysis was determined by measuring total weekly urea clearance (\(Kt/V\), where \(K\) is a constant, \(t\) is time, and \(V\) is total body water) and creatinine clearance by using standard methods (18). Weekly creatinine clearance was normalized to 1.73 m² of body surface area. The contribution of urea clearance by PD was estimated separately. The residual glomerular filtration rate was calculated as an average of 24-h urinary urea and creatinine clearance (19). The dialysate creatinine concentration was corrected for interference by glucose according to the reference formula determined in our laboratory (20). Total body water was derived from the Watson formula (21). Normalized protein-equivalent nitrogen appearance was calculated by the method described by Randerson et al (22) and was normalized to actual body weight.

Venous blood was collected on the morning of nutritional assessment for measurement of high-sensitivity C-reactive protein (hs-CRP), serum urea, creatinine, and albumin. CRP was measured by using the Tina-quant CRP (Latex) ultrasensitive assay (Roche Diagnostics GmbH, Mannheim, Germany). Serum albumin was measured by using the bromcresol purple method (23).

**Study outcome**

Patients were prospectively followed up after the assessments made at study baseline. No patient was lost to follow-up. Patients who underwent kidney transplants or who were transferred to hemodialysis were censored at the time of transfer to alternative renal replacement therapy. If a patient died within 3 mo of transfer to hemodialysis, he or she was not censored because the early mortality was considered to reflect the patient’s health status during the period of failing PD treatment. During the follow-up period, all deaths were accurately recorded. The exact primary event leading to the patient’s death was taken as the final cause of death and was provided by the attending physician, who had no knowledge of the HGS results. In the case of death out of hospital, family members were interviewed by telephone to ascertain the circumstances surrounding death. The clinical outcomes evaluated were all-cause and cardiovascular mortality. Cardiovascular mortality included death associated with a definite myocardial ischemic event, heart failure, cerebrovascular accident, arrhythmia, and peripheral vascular accident, all of which were defined by using standard clinical criteria. Sudden death was defined as unexpected natural death within 1 h of the symptom onset and without any prior condition that would appear fatal (24, 25).

**Statistical analysis**

Continuous data are presented as means ± SDs or medians (with interquartile ranges), and categorical data are presented as percentages. Comparisons between 2 groups were done by \(t\) test,
RESULTS

The baseline characteristics of the study population as a whole and for the men and women separately are shown in Table 1. The cause of end-stage renal disease was chronic glomerulonephritis in 76 patients (32.6%), diabetic nephropathy in 56 patients (24.0%), hypertensive nephrosclerosis in 31 patients (13.3%), obstructive uropathy in 13 patients (5.6%), polycystic kidney disease in 12 patients (5.2%), and tubulointerstitial disease in 6 patients (2.6%), and not identified in 39 patients (16.7%). None of the patients had active wrist or hand arthritis at the time of HGS assessment.

The correlation between age and HGS in the men and the women is shown in Figure 1. In the group as a whole, HGS was more strongly correlated with LBM by CK (Figure 2A) with than serum albumin (Figure 2B). With sex stratification, the correlation between HGS and serum albumin was greater in the men than in the women (Figure 2B).

The HGS of patients graded as being well nourished, mildly malnourished, and moderately or severely malnourished according to SGA was 21.5 ± 10.5, 17.9 ± 10.3, and 18.1 ± 11.1 kg, respectively (P = 0.043 for the overall difference between groups by one-way analysis of variance). Lower HGS was observed in persons with diabetes than in those without (15.9 ± 8.9 compared with 21.6 ± 10.8 kg; P < 0.001) and in patients with atherosclerotic vascular disease (defined as the presence of ischemic heart disease; history of angina, previous myocardial infarction, or coronary artery bypass surgery or angioplasty; or history of a cerebrovascular event, transient ischemic attack, or peripheral vascular disease with or without amputation) than in those without (16.8 ± 9.9 compared with 20.1 ± 10.7 kg; P = 0.018). On the other hand, HGS was greater in patients with residual renal function than in those without (20.2 ± 9.8 compared with 15.0 ± 9.5 kg; P < 0.001; Figure 1B). Other nutritional indexes, including serum albumin (29.3 ± 5.2 compared with 27.8 ± 4.9 g/L; P = 0.013) and LBM by CK (33.7 ± 7.4 compared with 29.1 ± 6.3 kg; P < 0.001) were also greater in patients with residual renal function than in those without. The prevalence of moderate and severe malnutrition according to SGA was greater among combined male and female patients with no residual renal function than among those with residual renal function (24.1% compared with 10.3%; P = 0.018).

The correlation analysis of HGS with different variables after adjustment for age, sex, and body height in the fully adjusted model are shown in models 1 and 2, respectively, in Table 2. HGS showed no correlation with CRP in either model. On the other hand, serum albumin was correlated with CRP (R = −0.200, P = 0.002; data not shown). The associations of the different nutritional indexes with HGS are shown in Table 3.

After being followed up for a mean duration of 30 ± 14 mo, 78 patients (40 men and 38 women, 33.5%) had died (autopsies were

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### Table 2

<table>
<thead>
<tr>
<th>Nutritional index</th>
<th>Partial correlation coefficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBM by CK</td>
<td>0.334</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>0.237</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SGA (maldnourished versus well nourished)</td>
<td>−0.200</td>
<td>0.002</td>
</tr>
<tr>
<td>nPNA</td>
<td>0.087</td>
<td>0.188</td>
</tr>
</tbody>
</table>

---

### Table 3

<table>
<thead>
<tr>
<th>Nutritional index</th>
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<tbody>
<tr>
<td>LBM, lean body mass; CK, creatinine kinetics; SGA, subjective global assessment; nPNA, normalized protein-equivalent nitrogen appearance. Adjusted for age, sex, and body height by using multiple regression analysis.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

### Table 4

<table>
<thead>
<tr>
<th>Causes of death of peritoneal dialysis patients</th>
<th>Total death (n = 78)</th>
<th>Men (n = 40)</th>
<th>Women (n = 38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular causes</td>
<td>51 (65.4)</td>
<td>25 (62.5)</td>
<td>26 (68.4)</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>6 (7.7)</td>
<td>2 (5)</td>
<td>4 (10.5)</td>
</tr>
<tr>
<td>Cardiac failure</td>
<td>3 (3.8)</td>
<td>2 (5)</td>
<td>1 (2.6)</td>
</tr>
<tr>
<td>Sudden cardiac death</td>
<td>23 (29.5)</td>
<td>13 (32.5)</td>
<td>10 (26.3)</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
<td>5 (6.4)</td>
<td>3 (7.5)</td>
<td>2 (5.3)</td>
</tr>
<tr>
<td>Arrhythmia</td>
<td>1 (1.3)</td>
<td>0 (0)</td>
<td>1 (2.6)</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>13 (16.7)</td>
<td>5 (12.5)</td>
<td>8 (21.1)</td>
</tr>
<tr>
<td>Noncardiovascular causes</td>
<td>27 (34.6)</td>
<td>15 (37.5)</td>
<td>12 (31.6)</td>
</tr>
<tr>
<td>Peritonitis</td>
<td>11 (14.1)</td>
<td>5 (12.5)</td>
<td>6 (15.8)</td>
</tr>
<tr>
<td>Other infections</td>
<td>10 (12.8)</td>
<td>6 (15)</td>
<td>4 (10.5)</td>
</tr>
<tr>
<td>Malignancy</td>
<td>1 (1.3)</td>
<td>0 (0)</td>
<td>1 (2.6)</td>
</tr>
<tr>
<td>Other causes, including termination of dialysis</td>
<td>5 (6.4)</td>
<td>4 (10)</td>
<td>1 (2.6)</td>
</tr>
</tbody>
</table>

1 Percentages may not add up to 100 because of rounding.
performed for 6), 29 patients (12.4%) underwent kidney transplantation, and 24 patients (10.3%) were changed to long-term hemodialysis. The causes of death in the men and the women are presented in Table 5. Cardiovascular causes accounted for 65.4% of all deaths in our patients, followed by peritonitis (14.1%) and other infections (12.8%). By excluding patients who underwent kidney transplantation or changed to long-term hemodialysis, we compared the baseline characteristics of the survivors (78) and nonsurvivors (102) 

**Table 5**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Survivors (n = 102)</th>
<th>Nonsurvivors (n = 78)</th>
<th>P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex [n (%)]</td>
<td>49 (48)</td>
<td>40 (51)</td>
<td>0.666</td>
</tr>
<tr>
<td>Age (y)</td>
<td>54 ± 12</td>
<td>61 ± 10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Duration of dialysis (mo)</td>
<td>24 (13, 49)</td>
<td>30 (14, 49)</td>
<td>0.144</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.0 ± 3.3</td>
<td>23.3 ± 3.7</td>
<td>0.522</td>
</tr>
<tr>
<td>Diabetes [n (%)]</td>
<td>28 (28)</td>
<td>33 (42)</td>
<td>0.037</td>
</tr>
<tr>
<td>History of smoking [n (%)]</td>
<td>34 (33)</td>
<td>35 (45)</td>
<td>0.115</td>
</tr>
<tr>
<td>Atherosclerotic vascular disease [n (%)]</td>
<td>13 (13)</td>
<td>30 (39)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum albumin (g/L)</td>
<td>29.9 ± 4.8</td>
<td>26.7 ± 4.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>1.58 (0.66, 5.12)</td>
<td>5.32 (2.07, 11.76)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Handgrip strength (kg)</td>
<td>19.7 ± 9.7</td>
<td>13.1 ± 8.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Subjective global assessment [n (%)]</td>
<td>69 (68)</td>
<td>33 (42)</td>
<td>0.002</td>
</tr>
<tr>
<td>Well nourished</td>
<td>22 (22)</td>
<td>26 (33)</td>
<td>0.006</td>
</tr>
<tr>
<td>Moderately or severely malnourished</td>
<td>11 (11)</td>
<td>19 (24)</td>
<td></td>
</tr>
<tr>
<td>LBM by CK (kg)</td>
<td>32.7 ± 7.9</td>
<td>29.8 ± 6.1</td>
<td>0.028</td>
</tr>
<tr>
<td>Peritoneal dialysis Kt/V</td>
<td>1.54 ± 0.37</td>
<td>1.52 ± 0.38</td>
<td>0.643</td>
</tr>
<tr>
<td>Residual GFR (mL · min⁻¹ · 1.73 m⁻²)</td>
<td>0.86 (0.20, 3.03)</td>
<td>0.02 (0.1, 1.29)</td>
<td>0.005</td>
</tr>
<tr>
<td>nPNA (g · kg⁻¹ · d⁻¹)</td>
<td>0.98 ± 0.18</td>
<td>0.92 ± 0.21</td>
<td>0.040</td>
</tr>
</tbody>
</table>

1 LBM, lean body mass; CK, creatinine kinetics; Kt/V, urea clearance; GFR, glomerular filtration rate; nPNA, normalized protein-equivalent nitrogen appearance. Percentages may not add up to 100 because of rounding.
2 P values for comparisons between survivors and nonsurvivors by t test for data expressed as x ± SD, by Mann-Whitney test for data expressed as median (interquartile range), or by chi-square test for categorical data.
3 x ± SD (all such values).
4 Median; interquartile range in parentheses (all such values).
5 Sex showed no significant interaction with all the variables above in relation to survivor status except with serum albumin (P = 0.048). Serum albumin was 30.1 ± 3.8 and 25.6 ± 4.9 g/L for male survivors and nonsurvivors, respectively (P < 0.001) and 29.6 ± 5.5 and 28.0 ± 3.9 g/L for female survivors and nonsurvivors, respectively (P = 0.112).

The men and women were separately stratified into tertiles according to their HGS: in men, the cutoffs were HGS ≤ 20.0 kg (lower), >20.0 but ≤ 29.0 kg (middle), and >29.0 kg (upper tertile); in women, the cutoffs were HGS ≤ 10.0 kg (lower), >10.0 but ≤ 18.0 kg (middle), and >18.0 kg (upper tertile). The Kaplan-Meier analysis in relation to the overall survival and cardiovascular-event-free survival of men and women in the 3 tertiles of HGS is shown in Figure 3A and B. There was no significant interaction between sex and tertiles of HGS for all-cause mortality (P = 0.904) or cardiovascular mortality (P = 0.658).

The univariate and multiple Cox regression analyses in relation to all-cause and cardiovascular mortality are detailed in Table 6. In the multiple Cox regression analysis, serum albumin and HGS were each independently associated with all-cause and cardiovascular mortality. HGS remained associated with all-cause and cardiovascular mortality when additional adjustment was made for serum albumin. However, serum albumin lost its significant association with cardiovascular mortality in the fully adjusted model. In contrast, with control for the same confounding covariates as listed in Table 6, SGA showed no associations with all-cause (P = 0.119 without control for serum albumin and P = 0.667 when also controlled for serum albumin) or cardiovascular (P = 0.485 without control for serum albumin and P = 0.901 when also controlled for serum albumin) mortality. LBM by CK also showed no independent associations with all-cause (P = 0.341 without control for serum albumin and P = 0.951 when also controlled for serum albumin) or cardiovascular (P = 0.864 without control for serum albumin and P = 0.401 when also controlled for serum albumin) mortality.

**DISCUSSION**

In the present prospective study, we showed that HGS was independently associated with all-cause and cardiovascular mortality in PD patients regardless of whether we controlled for serum albumin. Compared with serum albumin, HGS appeared to be slightly less significant in predicting all-cause mortality but was more strongly associated with cardiovascular death in PD patients. The lack of an independent association between serum albumin and cardiovascular death may be partly explained by serum albumin’s well-known correlations with CRP and cardiovascular disease and that both CRP and cardiovascular disease were more powerful predictors of cardiovascular mortality in PD patients (26). Indeed, even though serum albumin is frequently considered a nutritional marker and has been shown to predict outcome in dialysis patients (5, 6, 9), there is increasing evidence that it may be more related to chronic inflammation than to
nutritional status (27, 28) and that the reported association between hypoalbuninemia and mortality may be due to inflammation rather than to poor nutritional intake. Hypoalbuninemia is also a marker of extracellular volume overload (29, 30) and is frequently associated with cardiac comorbidity (14, 31). As shown several studies (14, 31), comorbidity may be more important than malnutrition in determining serum albumin in patients with end-stage renal disease. On the other hand, hypoalbuninemia is also related to increased peritoneal (32) and urinary protein loss (11). Thus, hypoalbuninemia is not simply a marker of malnutrition but also reflects underlying inflammation and comorbidity. It becomes obvious that we need a nutritional marker that more reliably reflects the nutritional status of PD patients. Furthermore, the bromocresol purple method underestimates serum albumin in dialysis patients (33), and the discrepancy is even higher among PD patients (34).

HGS is a measure of body protein loss and predicts mortality and complications in surgical patients (12, 35) and in the elderly (36, 37). It has also been reported to correlate with nutritional indexes, including fat-free mass in chronic heart failure patients (38), arm muscle area in the elderly (39), and SGA in hemodialysis patients (1). In a previous study by Heimburger et al (11), HGS was strongly correlated with LBM in chronic renal failure patients. However, no association was observed between HGS and serum albumin. Another study by Jones et al (30) also showed no association between serum albumin and HGS or any other nutritional index in patients undergoing continuous ambulatory PD.

In the present study of PD patients, HGS showed the strongest correlation with LBM estimated by CK, followed by serum albumin and SGA. This in keeping with previous studies in other renal failure populations (11, 29) and indicates that HGS is a good marker of body lean muscle mass and nutritional status in PD patients. Of more importance was that unlike serum albumin, HGS is not confounded by the presence of inflammation, suggesting that HGS is a better and more reliable nutrition marker in PD patients than is serum albumin. At present, there is no gold standard for assessing nutritional status in dialysis patients. Malnutrition is usually defined on the basis of changes in several different nutritional indicators, including clinical, biochemical, and anthropometric measurements. A previous study by Heimburger et al (11) showed that HGS had the strongest independent association with malnutrition assessed by using SGA. In our study, HGS was the only nutritional marker that consistently showed a significant difference between survivors and nonsurvivors for both men and women. Other nutritional markers, including serum albumin, SGA, and LBM by CK showed significant differences in men but not in women. That finding suggests that HGS may be a better nutritional marker in differentiating outcomes in both male and female PD patients.

Apart from age and sex (12, 14, 35), factors such as patient motivation, body position (40), and muscular weakness caused by uremia can also affect HGS. In our study, diabetes and atherosclerotic vascular disease were associated with lower HGS, suggesting indeed an important association between comorbidity and malnutrition. HGS was also greater among those with residual renal function than among those with no residual renal function. This finding agrees with the results for other nutritional indexes and suggests that the patients with residual renal function had better nutritional status. PD patients who were more anemic also had lower HGS, which may have been partly attributable to poor muscle strength and not just to a more malnourished state. It was intriguing that 5 women and 1 man failed to achieve a HGS greater than zero despite 3 attempts; this may relate to very low LBM together with extremely poor muscle strength. These patients did follow the instructions given and performed the HGS test in the proper way.

In contrast with HGS, LBM by CK and SGA showed no independent association with all-cause and cardiovascular mortality. This indicates that HGS is not only a more reliable nutrition marker but also a more significant predictor of outcome in PD patients. Both SGA and LBM by CK have their limitations. SGA is subjective and that limits its reproducibility. Studies have also reported marked underestimation of LBM by using CK compared with other methods, such as total body potassium and anthropometry (41, 42). Furthermore, assumptions about extra-renal creatinine metabolism and incompleteness of urine and dialysate collection may affect the accuracy of estimation of LBM by CK. In contrast, HGS is a simple, quick, easily performed and inexpensive bedside test. It has the additional
The advantage of not being affected by the hydration state of patients or by inflammation, unlike serum albumin.

This study had several limitations worth considering. First, our study included only patients who had been receiving PD treatment for some time, which may introduce a survival bias. Second, HGS and other nutritional indexes were all measured at a single time point and thus do not reflect changes over time. Further prospective study with serially measured HGS is needed.

In conclusion, our results showed that HGS represents a regular nutrition-monitoring tool in PD patients.

AY-MW contributed to the concept of the study, study design and data collection, data analysis, and writing of the manuscript. MM-MS contributed to data collection. ZS-YH contributed to data collection. S-FL contributed to the study design and review of the manuscript. PK-TL contributed to the study design and review of the manuscript. JW contributed to the idea of the study, study design, and final review of the manuscript and is the principal investigator of the Bristol Myers Squibb Unrestricted Nutrition Grant Program. None of the contributing authors had any financial or personal interest in any company or organization sponsoring the research.

REFERENCES


Postprandial metabolic utilization of wheat protein in humans1–3

Cécile Bos, Barbara Juillet, Hélène Fouillet, Lucie Turlan, Sophie Daré, Catherine Luengo, Rufin N’tounda, Robert Benamouzig, Nicolas Gausserès, Daniel Tomé, and Claire Gaudichon

ABSTRACT

Background: The quality of cereal protein has been little studied in humans despite its quantitative importance in the diet, particularly in developing countries.

Objective: The objective of this study was to determine the nutritional value of wheat protein in humans as assessed by the measurement of their real ileal digestibility and postprandial retention.

Design: Healthy young adults (n = 14) were fitted with an intestinal tube to allow the collection of intestinal fluid in the duodenum or terminal ileum. Subjects received a mixed meal of 136 g wheat toast that contained 24.6 g uniformly and intrinsically [15N]-labeled wheat protein. Intestinal fluid, blood, and urine were collected for 8 h postprandially.

Results: The real ileal digestibility of dietary wheat nitrogen amounted to 90.3 ± 4.3%. The cumulative amount of dietary nitrogen transferred to the deamination pools reached a plateau at 8 h of 24.7 ± 6.8% of the amount ingested. The urinary excretion of dietary nitrogen in ammonia was high (0.8 ± 0.3% of ingested dose). The incorporation of dietary nitrogen into serum protein reached 7.0 ± 1.9% of the meal. Postprandial wheat protein retention was 66.1 ± 5.8%.

Conclusions: Our results show that wheat proteins had the same true ileal digestibility as did most of the plant proteins already studied in humans, but also that they had a lower postprandial nitrogen retention value. However, this low value was higher than that predicted from the calculation of indispensable amino acid scores, ie, 89% rather than 30–40% of the nutritional value of milk proteins. Am J Clin Nutr 2005;81:87–94.

KEY WORDS: Nitrogen metabolism, dietary nitrogen, wheat protein, nonsteady state, urea production, protein quality, humans

INTRODUCTION

Cereals are an important dietary protein source throughout the world, because they constitute the primary protein and energy supply in most developing countries. Cereals also make a significant contribution (of nearly 20%) to the daily protein intake in developed countries, mainly in the form of bread, pasta, rice, and breakfast cereals (1).

Wheat contains moderate amounts of protein (8–12% on a weight basis) mainly composed of storage proteins or gluten proteins (80–85% of total wheat protein), classified as gliadins and glutenins. These are also referred to as "prolamins" because of their high content in glutamine and proline, which occurs at the expense of indispensable amino acids (AAs); particularly lysine, and to a lesser extent threonine. This lysine deficiency is a common feature of all cereal proteins, with values of 22–35 mg/g protein in wheat, 30–36 mg/g protein in rice, and 28–42 mg/g protein in maize. Although the lysine intake is evaluated at ~100 mg · kg⁻¹ · d⁻¹ in a typical Western diet, this intake only attains 44 mg · kg⁻¹ · d⁻¹ in British vegetarians and is likely to be much lower in countries with a cereal-based diet (2). In well-nourished young Indians, a habitual daily lysine intake of 53 mg · kg⁻¹ · d⁻¹ was recorded, but this intake falls to 39 mg · kg⁻¹ · d⁻¹ when measured in chronically undernourished Indian men (3, 4). Depending on the method used, lysine requirements in adults range from 12 to 45 mg · kg⁻¹ · d⁻¹ (3–9). Thus, because of their low protein and lysine content, covering protein and lysine requirements of subjects eating predominantly cereal-based diets is an important public health issue.

The capacity of wheat protein to fulfill the protein requirements of humans was traditionally determined by using nitrogen balance methods in both humans and animals (10, 11). Although a valid method, it is not the most sensitive in assessing protein nutritional quality. In the late 1980s, the FAO/WHO proposed the limiting indispensable AA scoring method for protein quality evaluation (12). On the basis of current data, the lysine content of dietary proteins which covers the lysine requirements at a safe level of 0.8 g protein · kg⁻¹ · d⁻¹ is within the range of 15–58 mg lysine/g protein, and under these conditions the quality indexes of gluten and whole wheat protein are 26–40%, respectively (13). More recently, a [13C]leucine balance protocol was applied to comparisons of wheat gluten and milk protein and showed that the wheat protein score was 61% that of milk proteins. However, these results were based on several assumptions, including the estimated AA availability of the 2 dietary proteins, and were derived from an indirect method because the nitrogen balances were extrapolated from leucine metabolism (9, 14).

To date, the ileal bioavailability and postprandial utilization of wheat protein were not addressed in humans, and the nutritional...

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value of wheat protein is still not clearly and directly determined, so that current opinions diverge, depending on the method used (9, 12, 13). For these reasons, during this study, we determined the quality of wheat protein by assessing its true ileal digestibility in healthy human subjects and its subsequent postprandial metabolism and retention by using intrinsically labeled wheat protein given in a single meal to healthy young adults equipped with an ileal tube.

SUBJECTS AND METHODS

Subjects

Fourteen subjects (6 women, 8 men) were enrolled in the study after a thorough medical examination and routine blood tests. Their mean age was 25 y and their body mass index (BMI; in kg/m²) was 21.3 ± 2.8 (Table 1). All subjects received detailed information on the protocol and gave written informed consent to their participation to the study. The protocol was approved by the Institutional Review Board for St-Germain-en-Laye Hospital, France.

Meals

The test meals comprised 136 g wheat toasts (in the form of biscoffes, a typical French breakfast ingredient resembling toast) that provide 24.6 g protein (298 mmol N), uniformly and intrinsically labeled with [15N] (enrichment: 0.6485 atom %). The [15N]-labeled wheat was prepared by ARVALIS, the Plant Institute (Institut du Végétal, Paris, France). [15N]-wheat flour was enriched with [15N]-gluten to produce wheat toast with the use of a standard recipe. Wheat biscuits were prepared by Danone, Centre Jean Thèves (Athis-Mons, France). The profile of protein fractions was determined by using the Profitabéle method (size exclusion-HPLC) as follows: 16% high-molecular-weight glutenin subunits; 24% low-molecular-weight glutenin subunits; 8% α-gliadins; 40% α-, β-, and γ-gliadins; and 12% albumins and globulins. The energy content of the meal was 2448 kJ of which 54.7% was carbohydrate (starch), 16.8% protein, and 28.5% fat.

Protocol

The subjects arrived at the hospital at 0900 the day before the experiment, in a fasted state (day 1). An intestinal tube was passed through the nose under local anesthesia and was allowed to progress through the digestive tract for the next 24 h by gravity and peristaltic contractions, as described previously (15). The subjects were given meals at 1200 and 1900 and then fasted overnight. Body composition was measured by using bioelectrical impedance analysis (Anlycor 5W; Eugened, Spengler, Cachein, France) in subjects lying at rest for ≥20 min. On the experimental day (day 2), the position of the tube in the intestine was checked under X-ray. In 9 subjects, the tube had correctly reached the terminal ileum (mean tube length from the nose: 186 ± 21 cm). In 5 subjects, the tube was stopped at the duodenum level (86 ± 5 cm from the nose) to assess the stomach delivery kinetics of the meal nitrogen. The protocol started at 1000, when a saline solution (130 mmol NaCl/L, 5 mmol KCl/L, 50 mmol d-mannitol/L) containing 400 mg phenol red/L (or phenolsulfonphthalein, PSP) was infused continuously through one lumen of the tube at a constant rate of 1 mL/min. The collection of a 30-min baseline sample of intestinal fluid started as soon as the marker (PSP) appeared in the second (collection) lumen (t0). At time zero, after a baseline blood sample and urine collection, the test meal was offered to the subjects. The meal was ingested within 30 min and was accompanied by 300 mL water. Intestinal fluid, blood, and urine sampling lasted for 8 h in the subjects with the ileal tube and for 6 h in the subjects with the duodenal tube. Digesta samples were collected over ice and pooled over 30-min periods. After volume measurement, samples were divided into aliquots and frozen at −20 °C. Blood was sampled every 30 min for 3 h and then every hour for the 5 remaining hours. Plasma and serum samples were obtained after centrifugation, divided into aliquots, and frozen at −20 °C. Total urine was collected every 2 h throughout the 8-h postprandial period. Urine specimens were weighed, divided into aliquots, and kept at −20 °C until analysis.

Analytic methods

The concentration of PSP in digesta samples was determined by using a colorimetric method (16). Plasma glucose was assayed by a glucose oxidase method (Glucose GOD-DP-kit; Kone, Evry, France). Urea concentrations were assayed in both serum and urine by using an enzymatic method on a Dimension automat (du Pont de Nemours, Les Ulis, France). Ammonia was measured in the urine by an enzymatic method on a Kone instrument (Kone). Amino acid concentrations were determined by HPLC with post-column ninhydrin derivatization (Biotek Instruments, St Quentin-en-Yvelines, France) in deproteinized serum samples. For isotopic determinations, urea and ammonia were isolated from urine as previously described, using a sodium and potassium form of the cation exchange resin (BioRad Dowex AG-50X8, mesh 100–200; Interchim, Montluçon, France) (17). Separation of nitrogen fractions (protein nitrogen, free nitrogen, and urea nitrogen) was achieved by adding 200 μL of a 1 g 5-sulfo-salicilic acid/mL solution to 4 mL serum. After 1 h at 4 °C, the samples were centrifuged (2400g for 20 min at 4 °C), and the pellet was dried and weighed for the quantification of total nitrogen in the protein fraction. The supernatant fraction was titrated to pH 7 by the addition of 1 mol NaOH/L and 0.1 mol NaH2PO4/L and transferred on 0.5 mL cation exchange resin, and the urea was hydrolyzed with 16 μL urease (680 U/mL; Sigma-Aldrich, Saint Quentin Fallavier, France) for 2 h at 30 °C. The supernatant containing serum-free nitrogen was collected and dried. Before isotopic determination, ammonia and urea-derived ammonia were eluted from the resin by the addition of 2.5 mol H2SO4/L. The total nitrogen content of the digesta and

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Subject characteristics</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Whole group (n = 14)</td>
</tr>
<tr>
<td>Gender (F/M)</td>
<td>6/8</td>
</tr>
<tr>
<td>Age (y)</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>65.1 ± 12.4</td>
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<tr>
<td>Height (m)</td>
<td>1.74 ± 0.10</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.3 ± 2.4</td>
</tr>
<tr>
<td>Fat-free mass (kg)</td>
<td>54.1 ± 12.4</td>
</tr>
<tr>
<td>Percentage of fat mass (%)</td>
<td>19.7 ± 8.3</td>
</tr>
<tr>
<td>Total body water (L)</td>
<td>37.3 ± 9.9</td>
</tr>
</tbody>
</table>

1 Body composition was assessed by bioelectrical impedance analysis.
2 x ± SD (all such values).
the serum protein fraction was determined by using an elemental nitrogen analyzer (NA 1500 series 2; Fisons Instruments, Manchester, United Kingdom) with atropina as standard. The isotope ratio of $^{15}$N to $^{14}$N was determined by isotope-ratio mass spectrometry (Optimal Fisons Instruments) in the digesta, urinary urea and ammonia, serum protein, free nitrogen, and urea. The atom percent excess (APE) of the samples was calculated by subtracting the baseline value from the atom percent determined at each time point.

**Calculations**

Data are expressed as means ± SDs. The total intestinal flow rate (Fi in mL/30 min) was derived for each 30-min period from the dilution of PSP, estimated by the following equation:

$$F = (PSP_i/PSPs) \times Fi \times t \quad (1)$$

where PSPi and PSPs are the PSP concentrations in the infusion solution and sample, respectively, Fi is the infusion rate (1 mL/min), and t is the collection duration (30 min).

The total nitrogen content of duodenal or ileal digesta (mmol nitrogen/30 min) was derived from the formula

$$N_{tot-digesta} = (N_i \times DM_i \times F)/140 \quad (2)$$

where $N_i$ is the nitrogen percentage measured (g/100 g) in the freeze-dried sample, DMi is the dry matter of the sample (g/100 mL), and F is the intestinal flow rate.

The time course of dietary nitrogen incorporation (expressed as a percentage of the ingested amount) into the different body nitrogen pools monitored (digesta, serum protein and free AA, body urea, urinary urea and ammonia) was evaluated by the following equation:

$$N_{diss}(t) = N_{tot}(t) \times [APE(i)/APE_{meal}] / N_{ingested} \times 100 \quad (3)$$

where $N_{tot}(t)$ is the total nitrogen content of the pool (in mmol nitrogen) at each time point t, APE(i) is the $^{15}$N enrichment above baseline in the nitrogen pool sampled at time t, APE_{meal} is the $^{15}$N enrichment of the meal, and N_{ingested} is the nitrogen content of the meal (in mmol nitrogen). The total nitrogen content in urinary urea and ammonia was obtained by multiplying the volume of urine by the corresponding urea and ammonia nitrogen concentrations. The total nitrogen content in the serum protein pool was calculated as the product of the nitrogen concentration in this fraction and the plasma volume, estimated at 5% of body weight (18). The total body urea nitrogen pool was calculated as the plasma urea nitrogen concentration multiplied by its volume of distribution (total body water) corrected by a factor of 92%, which represents the water content of blood.

The cumulated recovery of dietary nitrogen in ileal samples ($\Sigma N_{diss-ileal}$) served to calculate the real ileal digestibility (RID; percentage of ingested nitrogen) of wheat protein:

$$RID = (N_{ingested} - \Sigma N_{diss-ileal}) / N_{ingested} \times 100 \quad (4)$$

Total urea production (mmol N · kg BW$^{-1}$ · 2 h$^{-1}$) was evaluated for the four 2-h periods after meal ingestion by the following equation:

$$UP_{tot \ t - (t + 2)} = (UU_{tot \ t - (t + 2)} + (BU_{tot \ t - (t + 2)} - BU_{tot \ t}) / BW \quad (5)$$

where $UU_{tot \ t - (t + 2)}$ is the cumulative amount of urinary urea excreted between time t and $t + 2$ h, $BU_{tot \ t}$ and $BU_{tot \ t - (t + 2)}$ represent the body urea pool sizes at t and $t + 2$ h, and BW is the body weight.

Urea production of dietary origin (UP_{diet in mmol N · kg BW$^{-1}$ · 2 h$^{-1}$}) was calculated for each 2-h period as follows:

$$UP_{diet \ t - (t + 2)} = (UU_{diet \ t - (t + 2)} + (BU_{diet \ t - (t + 2)} - BU_{diet \ t}) / BW \quad (6)$$

where $UU_{diet \ t - (t + 2)}$ is the cumulative amount of dietary nitrogen excreted in urinary urea between time t and $t + 2$ h, and $BU_{diet \ t}$ and $BU_{diet \ t - (t + 2)}$ represent dietary nitrogen in the body urea pool at t and $t + 2$ h.

Endogenous urea production (UP_{endo in mmol N · 2 h$^{-1}$ · kg$^{-1}$}) was estimated for each 2-h period from the difference between total urea and dietary urea productions:

$$UP_{endo \ t - (t + 2)} = UP_{tot \ t - (t + 2)} - UP_{diet \ t - (t + 2)} \quad (7)$$

At the end of the 8-h experimental period, the amount of dietary nitrogen retained in the body and/or postprandial protein utilization (NPPU) was calculated as follows:

$$NPPU(% \ of \ ingested \ nitrogen) = (Meal \ nitrogen \ intake \ \times \ RID/100 - UP_{diet}) / Meal \ nitrogen \ intake \ (8)$$

The postprandial biological value (PBV) was calculated as the relative amount of dietary nitrogen absorbed that was not deaminated during the postprandial period:

$$PBV(% \ of \ ingested \ nitrogen) = NPPU/RID \times 100 \quad (9)$$

Because of the large volume of intestinal fluid collected in subjects with the tube positioned at the duodenal site (representing 10% of ingested nitrogen), the systemic data for these subjects were not used.

**Curve fitting**

The time courses of dietary nitrogen transfer into body nitrogen pools were fitted by using equations of the $y = a \cdot e(-1/2(\ln(\theta) / c)^2)$ type for body urea nitrogen pool and by equations of the $y = a \cdot [1-e(-c(t-\theta)^2)]$ type for serum-free nitrogen and protein-bound nitrogen, urinary urea, and urinary ammonia nitrogen (SIGMAPLOT software, version 6.00; SPSS Inc, Chicago).

**RESULTS**

The intestinal liquid flow rate and nitrogen kinetics after the meal are depicted in **Figure 1**. At the duodenal site, the intestinal fluid flow rate peaked early (370 mL/30 min), 1 h after the meal, whereas in the ileum, the liquid flow rate was constant, with a mean of 60 mL/30 min. In the duodenum, dietary nitrogen peaked at 1 h and 2.5 h after the meal and was the predominant component of nitrogen transit (64–77% between 1 and 4 h). In the ileum, the dietary nitrogen flux reached its maximum 4.5 h after the meal. The proportion of dietary nitrogen to total nitrogen averaged 28%. The half-time of dietary nitrogen transit through the duodenum was 117 ± 20 min. At the end of the experimental period, the cumulated amounts of dietary nitrogen which had
transited through the duodenum and ileum were 75.8 ± 17.4% and 9.7 ± 4.3% of ingested nitrogen (Figure 1). If the amounts were compared, 24.2 ± 17.4% of ingested nitrogen was absorbed at the duodenal level, and the RID of wheat nitrogen was 90.3 ± 4.3%.

Plasma glucose exhibited a two-phasic postprandial profile consistent with the intestinal nitrogen kinetics, with a first peak at 1 h and a second peak 2.5 h after the meal (Figure 2). In contrast, serum total amino acid concentration was only slightly increased 1 h after the meal and then decreased below the baseline amount. Serum lysine concentration decreased for the first 3 h after the meal from its fasting concentration (220 ± 92 mol/L) to 140 ± 92 mol/L and was stable thereafter (Figure 2).

Time courses of isotopic [15N] enrichments were measured in intestinal, systemic, and urinary nitrogen pools (Figure 3), which allowed for the quantification of dietary nitrogen in each pool. The incorporation of dietary nitrogen into the serum-free AA pool increased during the first 3 h after meal ingestion, reached a maximum of 0.56% of ingested nitrogen, and then slowly and linearly declined (Figure 4). The time course of dietary nitrogen incorporation into the serum protein pool was characterized by a sigmoid shape and a plateau that was reached after 7 h, with a value of 7.0 ± 1.9% of the ingested amount.

Urea and ammonia pools were also monitored to measure the time course of wheat-derived AA deamination (Figure 5). Dietary nitrogen transfer into body urea increased during the first 3 h and reached a quasi-plateau from 3 to 5 h after the meal, peaking at 16.5 ± 4.4% of the ingested nitrogen, and then declining slowly for the last 3 h to 11.9 ± 5.0%. In parallel, the excretion of dietary nitrogen in urinary urea increased during the postprandial period and reached 11.0 ± 3.4% at 8 h, which represented 65% of the plateau value, as indicated by curve fitting (y = 16.9[1−exp^{−0.26t}]^{3.33}). Thus, the total transfer of dietary nitrogen to urea at 8 h was 23.6 ± 6.3% of ingested nitrogen. The transfer of dietary nitrogen into urinary ammonia occurred regularly throughout the postprandial period and almost reached the plateau value at 8 h, with 0.8 ± 0.3% of ingested nitrogen recovered in this pool (Figure 5).

The rate of both endogenous and exogenous (dietary) urea production was computed for 2-h periods after meal ingestion (Figure 6). Urea production of dietary origin reached its maximum during the first 2 h (0.54 ± 0.22 mmol N·kg⁻¹·2 h⁻¹) and decreased over the next 4 h. It was null over the last 2 h of the postprandial period. Endogenous urea production was relatively constant throughout the postprandial period.

In summary, the metabolic utilization of dietary nitrogen after the ingestion of a wheat meal was characterized by losses of 33.9 ± 5.8% (9.7% ileal losses and 24.7% deamination losses) and by a retention, or net postprandial protein utilization, of 66.1 ± 5.8% (Table 2). Postprandial biological value reached 72.9 ± 6.9%. Cumulated total (ie, endogenous and exogenous) nitrogen losses were 413 ± 118 mmol 8 h after the wheat meal.
Dietary nitrogen losses through the ileum represented 30% of total ileal losses. With respect to deamination losses, the contribution of dietary nitrogen to total losses was 22% throughout the 8 postprandial hours (Figure 7). Cumulated nitrogen losses equalled the amount provided by the meal 5 h after ingestion. Consequently, 8 h after the meal, subjects were in a negative nitrogen balance, reaching 106 mmol.

DISCUSSION

This work constitutes the first complete and direct characterization of wheat protein bioavailability and utilization in healthy humans, as measured after a single meal containing intrinsically [15N]-labeled proteins, with sequential monitoring of dietary nitrogen transit through intestinal, serum, and urinary nitrogen pools. The RID of wheat proteins reached 90%, which is a standard amount for the digestibility of plant proteins. The metabolic utilization of absorbed, wheat-derived AA was directed toward deamination for 25% of the amount ingested. The net postprandial retention of wheat protein was then calculated as 66%.

Real wheat nitrogen ileal digestibility

The RID of wheat protein nitrogen was found to reach 90.3%, which is close to that of other plant proteins measured by using the same method in humans: 91.5% and 90% for soy and pea protein isolates, 89% and 91% for pea and sweet lupin flours, respectively (17, 19–21). The value obtained fell within the large data scale of true wheat ileal digestibility obtained in pigs or minipigs (83–95%) or using the rat balance method (91–93%) (13, 22–26). A critical point of our study was whether the global value for nitrogen reflected individual AA digestibilities (27). When both were measured in animals being fed wheat, it appeared that lysine was up to 14% less digestible than nitrogen (23, 25, 26). As a result, the availability of wheat protein-limiting AA may be lower than the availability of total nitrogen, with possible consequences on further metabolic utilization.

The digestibility of wheat protein nitrogen was “standard” for well-processed plant proteins, consistent with the lack of any antinutritional factors or digestion-resistant protein fractions. However, the present estimate of wheat protein digestibility was made under conditions of optimum digestion, including the refining of wheat flour, the absence of added fiber from the meal, and moderate heat treatment, the latter being likely to improve protein hydrolysis by intestinal proteases. As a result, the value
found may be superior to that applicable to wheat products consumed in other parts of the world where higher contents in fibers and antinutritional factors lower the true protein digestibility values (12, 28).

Wheat nitrogen postprandial utilization

The postprandial metabolism of wheat protein was characterized by a higher deamination of wheat-derived AA (25% of ingested nitrogen over 8 h) than the amount of 16–20% measured for other protein sources including milk, soy, lupin, or pea (19–21, 29), which indicated a lower retention of wheat protein. Our results indicate that, in vivo and under the physiologic conditions of meal ingestion, the postprandial retention of wheat protein nitrogen was 89% that of milk protein and 94% that of soy protein (30), which is higher than the relative nutritional values of gluten and milk protein found previously (61%) (9, 14). Moreover, our results argue strongly in favor of revising the protein quality indexes on the basis of the ratio between the more limiting AA and that of an AA pattern considered as a reference (ie, protein digestibility–corrected AA score; PD-CAAS; 12). Gluten PD-CAAS values reach 25%, and those of whole-wheat protein reach 40–42%; the latter represents 45% and 34% of soy and milk protein (untruncated) PD-CAAS, respectively (13, 31). Although PD-CAAS values are likely to change with the reassessment of indispensable AA requirements, this method clearly penalizes protein sources deficient in AA, as was already observed for the globulin fraction of pea protein (20).

Our findings, showing higher wheat protein retention than expected, may also suggest that well-nourished subjects have the ability to buffer an acute low dietary supply of lysine. Because the habitual occidental diet of the subjects presumably contained excess lysine and protein, and because lysine is an indispensable
amino acid with large body stores (32, 33), it is possible that the metabolic utilization we measured reflects a maximum value. Nonetheless, not only serum lysine but also total serum amino acid amounts decreased throughout the postprandial period, which is not a typical postprandial response to a protein meal (32, 34). This observation suggests that there was an acute effect of lysine deficiency on peripheral amino acid availability, possibly limiting protein synthesis. Further investigations are required in subjects chronically exposed to sublimiting lysine amounts to assess the consequences of such deficiencies on wheat protein metabolic utilization.

As for the metabolic utilization of dietary AA for synthetic purposes, the incorporation of dietary nitrogen into the serum protein pool after ingestion of the wheat meal was 7%, which compares better with the amount found after milk protein ingestion than after soy protein ingestion (30, 34). The kinetics of nitrogen absorption is known to strongly influence the degree to which dietary AAs are dispatched through the anabolic and catabolic pathways. The profile of dietary incorporation into the serum AA pool was as fast as that observed after soy protein but was associated to a lower incorporation of dietary nitrogen into plasma protein. This suggests that protein synthesis efficiency might be actually limited in the case of wheat protein ingestion by the lysine deficiency despite the fact that subjects had probably high intracellular lysine stores.

TABLE 2
Summary of the bioavailability and postprandial metabolic utilization of wheat protein nitrogen 8 h after its ingestion in a single mixed meal in humans.

<table>
<thead>
<tr>
<th>Percentage of ingested nitrogen</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ileal losses</td>
<td>9.7 ± 4.3</td>
</tr>
<tr>
<td>Real ileal digestibility</td>
<td>90.3 ± 4.3</td>
</tr>
<tr>
<td>Deamination</td>
<td></td>
</tr>
<tr>
<td>Body urea</td>
<td>11.9 ± 5.0</td>
</tr>
<tr>
<td>Urinary urea</td>
<td>11.0 ± 3.4</td>
</tr>
<tr>
<td>Urinary ammonia</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>Total deamination losses</td>
<td>24.7 ± 6.8</td>
</tr>
<tr>
<td>Total losses</td>
<td>33.9 ± 5.8</td>
</tr>
<tr>
<td>Net postprandial protein utilization</td>
<td>66.1 ± 5.8</td>
</tr>
<tr>
<td>Postprandial biologic value</td>
<td>72.9 ± 6.9</td>
</tr>
</tbody>
</table>

All values are x ± SD.

Lysine requirements and wheat protein quality in human diets

It is difficult to assess the extent to which lysine deficiency of cereal products is limiting in cereal-based diets and has any effect on functional and immune status, growth, and body composition. A broad range of lysine requirements (12–45 mg · kg⁻¹ · d⁻¹) was reported, but, according to the most recent data, the lower value of 12 mg · kg⁻¹ · d⁻¹, which corresponds to the latest international recommendations published (8), is markedly underestimated. The true value for lysine requirements is thus thought to lie between 18 and 45 mg · kg⁻¹ · d⁻¹, as assessed by using isotopic methods (3–7, 9, 14). Interestingly, the present study showed that an experimental wheat-containing meal providing 300 mmol N and 8.3 mg lysine/kg balanced postprandial nitrogen losses for the 5 h after ingestion (Figure 7). If lysine is considered to be the first limiting AA in wheat protein, this result may translate to a lysine requirement of 1.6 mg · kg⁻¹ · h⁻¹—ie, 40 mg · kg⁻¹ · d⁻¹. This value is derived from fed state measurements and as such probably overestimates the requirement. On the basis of the total nitrogen losses of 413 mmol at 8 h (Figure 7), the approximate lysine requirement would then be 1.4 mg · kg⁻¹ · h⁻¹, or 33.6 mg · kg⁻¹ · d⁻¹. This value would reach 31.6 mg · kg⁻¹ · d⁻¹ if corrected for a 25% recycling of ileal nitrogen (35). Although limited by the habitual high lysine intakes of the subjects, which probably led to the determination of an optimal wheat protein utilization and underestimated the derived lysine requirement, this value is consistent with published data.

On the basis of the postprandial utilization in humans, wheat protein (66% retention) was of lower nutritional quality than were milk (74%), soy (71%), pea (70%), and lupin (74%) proteins (20, 21, 30). To appreciate the incidence of the moderate nutritional value of wheat protein, it is crucial to consider the extent to which lysine deficiency is compensated for by other sources. All cereal-based diets include other types of protein, which are likely to compensate for the lysine deficiency of cereal proteins. It has long been acknowledged that adding lysine to gluten or wheat protein improves their biological value (10, 11, 36). It was also reported that subjects receiving for 50 d a predominantly (90–95%) wheat-based diet were able to maintain their nitrogen equilibrium but required a marked increase in their daily energy intake to achieve this (37). Such a diet was also shown to produce significant changes in the ratio of plasma indispensable AA to dispensable AA (38). The modalities of supplementation of cereal proteins with lysine-rich protein sources, if necessary, are not yet elucidated.

In conclusion, we showed that wheat protein has a RID within the current range of the values found for other plant protein sources but has a lower postprandial biological value. However, the relative deficit of net postprandial protein utilization of wheat protein nitrogen compared with milk protein was not as depressed as might be predicted from the ratios of lysine content between dietary proteins,
which confirmed that AA score-based methods are problematic in assessing protein nutritional quality.

We are indebted to Michel Leuillet, Christine Bar-L’Helgouac’h, and Alain Fontaine (ARVALIS) for constructive discussions and analyses of wheat protein. We also thank the staff of the Gastroenterology Unit at the Avicenne Hospital in Bobigny and the volunteers who participated in the study.

CB collected and analyzed the data and wrote the manuscript. BJ, LT, SD, and CL collected and analyzed the data. HF analyzed the data. RN and RB were responsible for the clinical management of the volunteers. NG provided the labeled wheat biscuits and contributed to manuscript preparation. DT and CG designed the study and participated in the writing of the manuscript. None of the authors had any financial or personal interest in any company or organization sponsoring the research.

REFERENCES
α-Tocopherol disappearance is faster in cigarette smokers and is inversely related to their ascorbic acid status¹–³

Richard S Bruno, Rajasekhar Ramakrishnan, Thomas J Montine, Tammy M Bray, and Maret G Traber

ABSTRACT

Background: Cigarette smokers have enhanced oxidative stress from cigarette smoke exposure and from their increased inflammatory responses.

Objective: The objective of this study was to determine whether cigarette smoking increases plasma α-tocopherol disappearance in otherwise healthy humans.

Design: Smokers and nonsmokers (n = 10/group) were supplemented with deuterium-labeled α-tocopheryl acetates (75 mg each of d₉-RRR-α-tocopheryl acetate and d₉-all-rac-α-tocopheryl acetate) for 6 evenings (days −6 to −1). Plasma α-tocopherols, ascorbic acid, uric acid, and F₂-isoprostanes were measured in blood samples collected on days −6 through 17. The urinary α-tocopherol metabolite, α-carboxy-ethyl-hydroxy-chroman (α-CEHC), was measured on days −6, 0, and 17 in 24-h urine samples.

Results: F₂-isoprostanes were, on average, ≈40% higher in smokers than in nonsmokers. On day 0, plasma labeled and unlabeled α-tocopherol concentrations were not significantly different between groups. Smoking resulted in faster fractional disappearance of plasma α-tocopherol (0.215 ± 0.011 compared with 0.191 ± 0.009 pools/d; P < 0.05). Fractional disappearance rates of α-tocopherol correlated with plasma ascorbic acid concentrations in smokers (P = 0.021) but not in nonsmokers despite plasma ascorbic acid concentrations that were not significantly different between groups. By day 17, cigarette smoking resulted in lower plasma α-tocopherol concentrations and urinary excretion of labeled and unlabeled α-CEHC (P < 0.05).

Conclusions: Cigarette smoking increased α-tocopherol disappearance. Greater rates of α-tocopherol disappearance in smokers appear to be related to increased oxidative stress accompanied by lower plasma ascorbic acid concentrations. Thus, smokers have an increased requirement for both α-tocopherol and ascorbic acid. Am J Clin Nutr 2005;81:95–103.

KEY WORDS Oxidative stress, vitamin E, vitamin C, cigarette smoke, antioxidants, dietary requirements

INTRODUCTION

Nearly 50 million Americans smoke cigarettes (1). The adverse health consequences of smoking have been largely attributed to the abundance of reactive oxygen species and reactive nitrogen species that readily react with various biomolecules. In fact, a single puff of cigarette smoke contains 1 × 10¹¹–¹² reactive oxygen species, ≈500 ppm nitric oxide, and other reactive nitrogen oxides (2). In addition to the stress of cigarette smoke, cigarette smokers also have increased inflammatory responses that further enhance their oxidative stress (3, 4).

Increased oxidative stress from cigarette smoke results in higher dietary ascorbic acid requirements for cigarette smokers (5). However, research to date has been unable to accurately define vitamin E dietary requirements or determine whether cigarette smoking or other oxidative stresses increase these requirements. As a result, the 2000 recommended dietary allowance for vitamin E is based largely on the in vitro assessment of hemolysis in erythrocytes obtained from experimentally caused vitamin E deficiency in men after treatment with peroxide (5–7).

Clearly, in vitro studies have indicated that cigarette smoke exposure depletes plasma α-tocopherol (α-T) concentrations (8–10). However, plasma α-T concentrations in humans trials (11–15) are often reported to not be significantly different between smokers and nonsmokers. Deuterium-labeled tocopherols have been used to determine the biokinetics, bioavailability, and metabolism of vitamin E in healthy humans and those with genetic abnormalities in lipoprotein metabolism and the α-T transfer protein (16–20). Unfortunately, attempts to characterize plasma α-T biokinetics in cigarette smokers with the use of deuterated tocopherols have not been entirely successful. Munro et al (21) supplemented smokers and nonsmokers on a single occasion with deuterated α-T and then collected blood samples 6, 12, and 27 h after the supplement. Although smokers had lower plasma deuterated α-T concentrations at each of the time points, it could not be elucidated whether these differences were due to reduced α-T absorption or its faster plasma clearance. Traber et al (22)

¹ From the Department of Human Nutrition, The Ohio State University, Columbus, OH (RSB); the Linus Pauling Institute, Oregon State University, Corvallis, OR (RSM, TMB, and MGT); the College of Physicians and Surgeons, Columbia University, New York (RR); the Department of Pathology, University of Washington, Seattle (TJM).
² Supported by grants to MGT (NIH DK59576) and TJM (AG05144 and AG16835). The Natural Source Vitamin E Association provided partial support for the purchase of the LC-MS and provided the deuterium-labeled α-tocopheryl acetate supplements. Tocopherol standards including d₁₀-γ-tocopherol were gifts from James Clark (Cognis Nutrition and Health, LaGrange, IL); all-rac-α-5,7,8-(CD₃)₃-tocopheryl acetate (d₉-all-rac-α-tocopheryl acetate) was provided by Carolyn Good (General Mills) and was synthesized by Isotec Inc (Miamisburg, OH).
³ Address reprint requests to MG Traber, 561A Weniger Hall, Linus Pauling Institute, Oregon State University, Corvallis, OR 97331. E-mail: maret.traber@oregonstate.edu.

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attempted to further clarify these differences by supplementing smokers and nonsmokers with deuterated α-T for 7 d and collecting fasting blood samples on select days up to day 21 after supplementation. Although smokers had a faster deuterated α-T disappearance, the findings were not statistically significant, likely because the investigation was underpowered.

Measurements of plasma and tissue α-T concentrations have been used to assess human vitamin E status. Plasma or urinary α-T metabolite (α-carboxyethyl-hydroxy-chroman; α-CEHC) concentrations may be another useful biomarker to assess vitamin E status. A nonoxidation product, α-CEHC is synthesized by hepatocytes via a cytochrome P450–dependent pathway before urinary excretion (23, 24). In humans, urinary α-CEHC is undetectable unless subjects are supplemented with sufficient α-T to surpass a 30–40 μmol/L plasma α-T threshold (25), which suggests that metabolism occurs when adequate or excessive hepatic α-T concentrations have been achieved.

In this investigation, we hypothesized that the higher magnitude of oxidative stress experienced by cigarette smokers than by nonsmokers would lead to more rapid plasma depletion of plasma α-T and to decreased urinary α-CEHC excretion. To test this hypothesis, we supplemented cigarette smokers and nonsmokers with deuterium-labeled α-T and measured the disappearance of plasma α-T and appearance of urinary α-CEHC using liquid chromatography–mass spectrometry (LC-MS) and gas chromatography–mass spectrometry (GC-MS), respectively.

SUBJECTS AND METHODS

Study participants

The protocol for this study was approved by the Institutional Review Board at Oregon State University, and all participants provided written consent before enrollment. Healthy, normolipidemic volunteers (n = 10 nonsmokers and 10 smokers) were selected for this study on the basis of age (18–35 y), nonnutritional supplement use (>6 mo), and exercise status (<5 h aerobic activity/wk). Participant characteristics are shown in Table 1. Nonsmokers (n = 6 men and 4 women) were selected on the basis that they had never smoked and did not reside with a smoker. Smokers (n = 6 men and 4 women) were selected if they smoked >10 cigarettes/d. Cotinine, the metabolite of nicotine, was measured by using a radioimmunoassay (Diagnostics Products Corp, Los Angeles). A urinary cotinine concentration of >500 ng/mL, as suggested by the manufacturer, was used as a cutoff to confirm smoking status.

To verify the participants’ health status before enrollment in the study, a serum chemistry profile (Table 2) was performed at Good Samaritan Regional Medical Center (Corvallis, OR). Additionally, hemoglobin was measured in whole blood with a kit, according to manufacturer’s instructions (Sigma Diagnostics, St Louis; procedure no. 525). Blood hematocrit was measured after 5 min of centrifugation (Statspin, Norwood, MA).

Dietary analysis

To control for potential confounders with respect to differences in dietary nutrient consumption, all participants completed a 3-d food record (2 weekdays and 1 weekend day) during the investigation. Dietary intakes were analyzed by using FOOD PROCESSOR (version 7.9; ESHA Research, Salem, OR).

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Participant characteristics at baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
<td>Nonsmokers</td>
</tr>
<tr>
<td>Age (y)</td>
<td>19.5 ± 1.5</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.69 ± 0.15</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>63.6 ± 14.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.2 ± 3.2</td>
</tr>
<tr>
<td>Nutrition supplements</td>
<td>None</td>
</tr>
<tr>
<td>Cigarettes smoked (no./d)</td>
<td>0</td>
</tr>
<tr>
<td>Urinary cotinine (ng/mL)</td>
<td>34 ± 13</td>
</tr>
<tr>
<td>α-Tocopherol (μmol/L)</td>
<td>15.3 ± 2.8</td>
</tr>
<tr>
<td>γ-Tocopherol (μmol/L)</td>
<td>1.63 ± 0.6</td>
</tr>
<tr>
<td>Ascorbic acid (μmol/L)</td>
<td>60 ± 22</td>
</tr>
<tr>
<td>Uric acid (μmol/L)</td>
<td>273 ± 57</td>
</tr>
<tr>
<td>F₂α-Isoprostanes (pg/mL)</td>
<td>30.6 ± 6.7</td>
</tr>
<tr>
<td>FRAP (μmol/L)</td>
<td>660 ± 106</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.34 ± 0.44</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.37 ± 0.17</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.42 ± 0.40</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.19 ± 0.55</td>
</tr>
</tbody>
</table>

Deuterated α-tocopherol

Capsules containing RRR-α-5-(CD₃)₆ and all-rac-α-5,7-(CD₃)₆ tocopheryl acetates (d₅-RRR-α-TAc and d₆-all-rac-α-TAc, respectively) were administered twice daily (5 capsule containing 100 mg α-T) to the study participants for 2 wk. Cigarette smokers and nonsmokers with deuterium labeled α-T and appearance of urinary α-CEHC (see Table 2) were selected for this study. All participants had blood chemistry values that were within the normal reference range.

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Blood chemistry and hematologic values of the subjects at screening</th>
</tr>
</thead>
<tbody>
<tr>
<td>Index</td>
<td>Normal range</td>
</tr>
<tr>
<td>Sodium (mEq/L)</td>
<td>135–145</td>
</tr>
<tr>
<td>Potassium (mEq/L)</td>
<td>3.5–5.1</td>
</tr>
<tr>
<td>Chloride (mEq/L)</td>
<td>106–111</td>
</tr>
<tr>
<td>Bicarbonate (mEq/L)</td>
<td>22–30</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>70–105</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>6–19</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.4–1.1</td>
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<tr>
<td>BUN:creatinine</td>
<td>6–30</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>8.4–10.2</td>
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<tr>
<td>Phosphorus (mg/dL)</td>
<td>2.7–4.5</td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
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<tr>
<td>Albumin (g/dL)</td>
<td>3.4–5.0</td>
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<tr>
<td>SGOT (U/L)</td>
<td>0–31</td>
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<tr>
<td>LDH (U/L)</td>
<td>94–250</td>
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<tr>
<td>SGPT (U/L)</td>
<td>0–31</td>
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<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>39–117</td>
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<tr>
<td>γ-GT (U/L)</td>
<td>7–33</td>
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<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.0–1.0</td>
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<td>Globulin (g/dL)</td>
<td>2.3–3.5</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.0–16.8</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>38–49</td>
</tr>
</tbody>
</table>

P = 0.007, *P = 0.030. Ferritin reducing ability of plasma.
respectively) were a gift from the Natural Source Vitamin E Association and were synthesized by Eastman Kodak (Rochester, NY). The d₃-RRR- and all-rac-α-TACs were encapsulated in a gelatin capsule as nominal 1:1 mixtures in 150-mg quantities. The molar ratio of d₃-RRR- to d₆-all-rac-α-T was determined to be 0.98 (26).

**Study protocol**

On 6 consecutive evenings, participants ingested the deuterated α-T supplement immediately after a standard meal. On average, this meal contained 1143 kcal (43% carbohydrate, 17% protein, and 41% fat), 35 mg ascorbic acid, and 2.7 mg α-T.

Blood samples were obtained after the subjects fasted overnight (10–12 h) on days −6, −5, −4, −3, −2, −1, 0, 1, 2, 3, 4, 5, 6, 8, 10, 13, 15, and 17 (negative days denote the supplementation period). Blood was collected from the antecubital vein into blood collection tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) containing 0.05 mL 15% K₂EDTA or sodium heparin. Smokers were asked to refrain from smoking for 1 h before blood collection to alleviate the transient oxidative stress effects.

Urine was collected for 24 h on 3 occasions: before supplementation period. Blood was collected from the antecubital vein into blood collection tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) containing 0.05 mL 15% K₂EDTA or sodium heparin. Smokers were asked to refrain from smoking for 1 h before blood collection to alleviate the transient oxidative stress effects.

Urine was collected for 24 h on 3 occasions: before supplementation period. Blood was collected from the antecubital vein into blood collection tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) containing 0.05 mL 15% K₂EDTA or sodium heparin. Smokers were asked to refrain from smoking for 1 h before blood collection to alleviate the transient oxidative stress effects.

**Materials**

Perchloric acid and HPLC-grade methanol were obtained from Fisher (Fair Lawn, NJ). The following were obtained from Sigma-Aldrich (St Louis): ascorbic acid, butylated hydroxytoluene, DTPA, FeCl₃, phosphate-buffered saline, potassium hydroxide, potassium phosphate trihydrate, TPTZ [2, 4, 6-tri(2-pyridyl)-s-triazine], and trolox. Chromatography pairing reagent, Q12 (1-dodecyltriethylammonium phosphate), was purchased from Regis (Morton Grove, IL). The isotopic purity of all-rac-α-T was found to be 88.4% d₉ and the remainder d₆.

**Tocopherol analysis**

Labeled and unlabeled tocopherols (Figure 1) were extracted according to procedures previously described (27) and were analyzed with an LC-MS that consisted of a Waters 2690 Separations Module (Milford, MA) and a ZQ 2000 instrument single-quadrupole mass spectrometer (Micromass, Manchester, United Kingdom), as previously described (11). The LC-MS was equipped with an atmospheric pressure chemical ionization probe set to the negative ionization mode. The mass-to-charge (m/z) ratios were as follows: d₉-α-T, m/z 429.4; d₆-α-T, m/z 432.4; d₆-α-T, m/z 435.4; d₉-α-T, m/z 438.4; and d₆-γ-T, m/z 415.4. Calibration curves were prepared with the use of authentic standards, and all-rac-α,β,γ,δ-CEHCs as unlabeled α- and γ-CEHCs (d₉-α- and d₉-γ-CEHC) and deuterium-labeled α- and γ-CEHCs (d₆-α- and d₆-γ-CEHC).

**Analysis of CEHCs**

Urinary deuterium labeled and unlabeled α- and γ-T metabolites (d₉-α-T, d₆-α-T, d₉-γ-CEHC and d₆-γ-CEHC; Figure 1) were measured by GC-MS according to a previously described method (28). The GC-MS consisted of an Agilent 6890 GC coupled with an Agilent 5973N MSD (Palo Alto, CA), and analyte concentrations were determined by electron impact ionization. In short, the urinary metabolites were extracted from 5 mL urine, derivatized with N,O-bis (trimethylsilyl) trifluoroacetamide and 1% trimethylchlorosilane, dried under nitrogen, and resuspended in hexane before injection. Sample volumes of 1 mL were injected onto the column with an HP 7683 auto-injector. Separations were performed on a DB-5MS column (30 m × 2.5 mm internal diameter, 25 μm film thickness; J & W Scientific, Folsom, CA) with helium as carrier gas. Quantification was performed by comparison with known amounts of an added internal standard (trolox).

**Ascorbic acid and uric acid analysis**

Ascorbic and uric acids were measured by HPLC-electrochemical detection as previously described (29). Standards of ascorbic acid were prepared fresh daily and verified spectrophotometrically with the ε265 nm of 14 500 M⁻¹ · cm⁻¹. Prepared uric acid standard was purchased from Pointe Scientific, Inc (Lincoln Park, MI).

**Ferritin reducing ability of plasma determination**

To assess plasma antioxidant capacity, the ferritin reducing ability of plasma (FRAP) was measured according to the methods of Benzie and Strain (30). In brief, 40 μL plasma (diluted 1:4) was mixed on a 96-well plate with 300 μL freshly prepared FRAP reagent (25 μL sodium acetate buffer (300 mmol/L), 2.5
Lipid analysis

Plasma $F_{2\alpha}-$isoprostanes were measured by GC-MS as previously described (31). Total cholesterol and triacylglycerol were measured with the use of the two-compartment model as previously described (22).

$$\%d_1-\alpha-T = [d_1-\alpha-T/(d_0-\alpha-T + d_1-\alpha-T + d_c-\alpha-T)] \times 100$$

(1)

The 2 compartments were assumed to have reached the same concentration at the end of 6 d of deuterated TAc supplementation. Fitting was performed by nonlinear least squares, assuming measurement error to have a constant CV. Fitting resulted in a fractional disappearance rate of $\alpha-T$ for each participant for which statistical comparisons could be made between nonsmokers and smokers. $\alpha-T$ half-lives were calculated as $\ln(2)/$disappearance rate constant.

Statistical analysis

Statistical analysis was performed with the use of GRAPHPAD PRISM (version 4.0; GraphPad Software, San Diego). An unpaired Student’s $t$ test was used for all comparisons between smokers and nonsmokers. All correlations were calculated by linear regression. To examine the effects of ascorbic acid concentrations and smoking on the fractional disappearance rates of $\alpha-T$, we conducted multiple linear regression analysis. All parameter estimates and $P$ values were obtained by using PROC GLM (version 8; SAS Institute Inc, Cary, NC). Data were considered statistically significant at $P < 0.05$. All data are reported as means ± SEs unless otherwise noted.

RESULTS

Participant characteristics and analysis of dietary intakes

There were no significant differences in age, height, weight, body mass index, plasma lipids, $\alpha-T, \gamma-T$, uric acid, or ascorbic acid at baseline between the smokers and nonsmokers (Table 1). However, smokers had a greater degree of oxidative stress, as marked by higher $F_{2\alpha}-$isoprostanes and lower FRAP. Participating cigarette smokers self-reported smoking between 10 and 20 cigarettes/d. Urinary cotinine values were correlated with the self-reported number of cigarettes smoked per day ($P = 0.010, R^2 = 0.582$) in the smokers. Nonsmokers had low or no measurable urinary cotinine concentrations (Table 1).

### TABLE 3

Dietary intakes of the nonsmokers and the smokers

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Nonsmokers ($n = 10$)</th>
<th>Smokers ($n = 10$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>2703 ± 498</td>
<td>2807 ± 773</td>
</tr>
<tr>
<td>Carbohydrate (% of energy)</td>
<td>50.5 ± 4.9</td>
<td>52.5 ± 2.2</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>15.4 ± 1.5</td>
<td>14.1 ± 2.3</td>
</tr>
<tr>
<td>Fat (% of energy)</td>
<td>35.0 ± 3.9</td>
<td>31.9 ± 5.3</td>
</tr>
<tr>
<td>Ascorbic acid (mg)$^2$</td>
<td>77.6 ± 25.3</td>
<td>73.4 ± 36.1</td>
</tr>
<tr>
<td>Vitamin E (mg $\alpha$-T)</td>
<td>5.5 ± 1.1</td>
<td>5.3 ± 2.2</td>
</tr>
</tbody>
</table>

$^1$ All values are $\bar{x}$ ± SD. Dietary intakes were estimated from 3-d food records. $\alpha-T, \alpha$-tocopherol. There were no significant differences between groups (unpaired Student’s $t$ test).

Surprisingly, no significant differences in baseline ascorbic acid concentrations were observed between nonsmokers and smokers (Table 1). Because it is often reported that cigarette smokers have lower plasma ascorbic acid concentrations than do nonsmokers (3, 33–35), these results could be due to a seasonal effect (36–38) because this study was conducted in early autumn. However, although these results for ascorbic acid status were unplanned, this may help isolate the effects of cigarette smoking on $\alpha-T$ disappearance kinetics. None of the participants took nutritional supplements for ≥6 mo before our study. Dietary intakes between smokers and nonsmokers were not significantly different for any of the nutrients analyzed (Table 3).

Plasma ascorbic and uric acids

After supplementation with deuterated $\alpha$-TAcS, plasma ascorbic acid concentrations averaged from days 0 to 17 were not significantly different between nonsmokers and smokers (Table 1). Plasma ascorbic acid concentrations were correlated with 3-d dietary ascorbic acid intakes (Table 3). The uric acid concentration, another major plasma antioxidant, was also not significantly different between the groups when averaged from days 0 to 17 (Table 4).

Ferritin reducing ability of plasma

FRAP is a measure of total antioxidant protection in plasma and is generally correlated with uric acid concentrations and to a

### TABLE 4

Plasma concentrations of lipids and antioxidant status markers after supplementation in the nonsmokers and the smokers

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Nonsmokers ($n = 10$)</th>
<th>Smokers ($n = 10$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.36 ± 0.61</td>
<td>3.78 ± 0.94</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/L)</td>
<td>1.00 ± 0.36</td>
<td>0.78 ± 0.29</td>
</tr>
<tr>
<td>Ascorbic acid (µmol/L)</td>
<td>52.1 ± 13.4</td>
<td>46.5 ± 17.8</td>
</tr>
<tr>
<td>Uric acid (µmol/L)</td>
<td>283.3 ± 41.3</td>
<td>278.2 ± 53.1</td>
</tr>
<tr>
<td>FRAP (µmol/L)</td>
<td>630 ± 56</td>
<td>554 ± 93$^2$</td>
</tr>
<tr>
<td>$F_{2\alpha}$-Isoprostanes (pg/mL)</td>
<td>32.4 ± 6.3</td>
<td>45.5 ± 16.0$^2$</td>
</tr>
</tbody>
</table>

$^1$ All values are $\bar{x}$ ± SD from days 0 to 17. FRAP, ferritin reducing ability of plasma.

$^2$ Significant difference from nonsmokers (unpaired Student’s $t$ test): $P = 0.021, \ P = 0.014.$
lesser extent with ascorbic acid concentrations (30). After supplementation, smokers had lower FRAP values than did nonsmokers (Table 4), which suggests that smokers had a lower plasma antioxidant status. When values were averaged from days 0 to 17 for each individual, FRAP correlated positively with uric acid concentrations (P < 0.0001, R² = 0.663) but not with plasma ascorbic acid concentrations (P = 0.53, R² = 0.023).

**Plasma isoprostanes**

To assess oxidative stress, F₂αα-isoprostanes were measured on selected days throughout the study. Cigarette smokers, on average, had F₂αα-isoprostanes that were ≈40% higher than those of nonsmokers throughout the postsupplementation period (Table 4). During the supplementation period, plasma F₂αα-isoprostanes decreased slightly in the smokers (≈13%) from days −6 (47 ± 5 pg/mL) to day 0 (41 ± 4 pg/mL), but these changes were not significantly different (P = 0.132).

**Tocopherols**

Before supplementation, plasma unlabeled α-T and γ-T concentrations were not significantly different between smokers and nonsmokers (Table 1). After 6 d of supplementation with 75 mg each of d₃-RRR-α-TACs and d₆-all-rac-α-TACs, plasma total α-Ts (sum of d₃-, d₆-, and d₆-α-T) more than doubled (P < 0.0001) in nonsmokers (from 15.3 ± 2.8 μmol/L at baseline to 35.5 ± 5.8 μmol/L on day 0) and in smokers (from 14.6 ± 3.8 μmol/L at baseline to 32.1 ± 10.2 μmol/L on day 0). Day 0 plasma concentrations of d₆-, d₆-, and d₆-α-T and total α-T were not significantly different between groups (Figure 2).

In both groups, plasma d₆-α-T concentrations decreased (P < 0.0001) by ≈19% in response to deuterated α-T supplementation, from 14.9 ± 0.5 μmol/L at baseline to 12.1 ± 0.8 μmol/L on day 0. Likewise, plasma γ-T concentrations decreased (P = 0.0011) by ≈38% in both groups, from 1.6 ± 0.1 μmol/L at baseline to 1.0 ± 0.1 μmol/L on day 0.

On day 0, plasma d₆-α-T concentrations in the nonsmokers (13.6 ± 1.2 μmol/L) and smokers (12.2 ± 1.5 μmol/L) were not significantly different, which suggested that both groups responded similarly to the 6 d of deuterium-labeled vitamin E supplementation (Figure 2). Likewise, plasma d₆-α-T concentrations were not significantly different between the nonsmokers (8.8 ± 0.8 μmol/L) and the smokers (8.6 ± 0.9 μmol/L). The ratio of d₆-α-T to d₆-α-T in the plasma was 1.5 ± 0.1 during the supplementation period and subsequently increased to 1.9 ± 0.1 after supplementation (days 0–17). These findings are similar to those from other human trials (16, 17, 39) that used deuterated RRR- and all-rac-tocopherols and, therefore, the subsequent results and discussion of α-T disappearance kinetics will be limited to d₆-α-T for simplicity.

After 6 d of deuterated α-T supplementation, the %d₆-α-T on day 0 did not differ significantly between groups (Figure 2), and d₆-α-T represented ≈40% of total plasma α-T. Mathematical modeling of the disappearance kinetics was performed on the %d₆-α-T data from days 0 to 17, and fitted data from a representative smoker and nonsmoker are shown in Figure 3. The fractional disappearance rates of α-T in cigarette smokers (0.215 ± 0.011) were ≈13% greater (P < 0.05) than those in nonsmokers (0.191 ± 0.009 pools/d; Figure 4). Likewise, calculated α-T half-lives were ≈10 h shorter in cigarette smokers (79.3 ± 4.1) than in nonsmokers (88.8 ± 3.8 h; P < 0.05).

**FIGURE 3.** Modeled curve fit and actual percentages of d₆-α-tocopherol (d₆-α-T) from days 0 to 17 in a representative smoker and nonsmoker. %d₆-α-T = [d₆-α-T/(d₆-α-T + d₃-α-T + d₆-α-T)] × 100.
μmol/L) of the smokers were lower (*P < 0.05*) than those of the nonsmokers (17.4 ± 1.2 μmol/L) by the end of the study. Despite these findings, there were no observable differences in d₆-α-T concentrations between smokers and nonsmokers before supplementation; moreover, dietary α-T intakes (as assessed with dietary food records during the study) were not significantly different between the groups.

It has been suggested that plasma tocopherol concentrations should be adjusted for circulating lipid concentrations (40). However, in this investigation, no significant differences in plasma total cholesterol or triglycerides were observed between groups on any of the study days (Table 4). Therefore, labeled and unlabeled tocopherol concentrations were not adjusted for total lipids.

**Urinary tocopherol metabolites**

The metabolites of α- and γ-T and α- and γ-CEHC, respectively, were measured on 3 occasions during the investigation in 24-h urine samples: at baseline (day −6), after 6 d of supplementation (day 0), and on the last day of the study (day 17). Given the limited data on α-T metabolism, we hypothesized that metabolite concentrations might serve as a marker for α-T status. As shown in Figure 5, there were no significant differences in unlabeled and labeled α-CEHC concentrations between the smokers and the nonsmokers at baseline or on day 0. Furthermore, the ratio of d₆-α-CEHC to d₃-α-CEHC on day 0 was 1.7 ± 0.1 in both groups, which indicated a greater conversion of d₆- all-rac-α-T to α-CEHC than did d₃-α-RRR-α-T. On day 17, 7 of 10 nonsmokers, but only 5 of 10 smokers, had detectable urinary deuterated α-CEHC concentrations. Therefore, for the undetectable values, a value of half the detectable limit was substituted for statistical analyses. We observed that smokers excreted less urinary d₆-α-CEHC (P < 0.05), d₃-α-CEHC (P < 0.05), and d₆-α-CEHCs (P = 0.09) than did nonsmokers. In addition, on day 17, plasma total α-Ts or urinary total α-CEHCS were lower among the smokers (Table 5). These data collectively support the notion that smokers had a lower α-T status at the end of the study.

No significant differences in γ-CEHC excretion were observed at baseline between the smokers (3.13 ± 0.54 μmol/g creatinine) and the nonsmokers (3.87 ± 0.86 μmol/g creatinine), on day 0 between the smokers (3.18 ± 0.70 μmol/g creatinine) and the nonsmokers (4.07 ± 0.89 μmol/g creatinine), or on day 17 between the smokers (2.05 ± 0.60 μmol/g creatinine) and the nonsmokers (3.44 ± 0.85 μmol/g creatinine).

**Interaction between α-T disappearance and ascorbic acid and uric acid**

Ascorbic acid is reported to regenerate α-tocopherol radicals to α-T in vitro (41), but this relation has not been shown in humans. Therefore, correlations were calculated between the α-T fractional disappearance rate and mean plasma ascorbic acid concentrations for each group. As shown in Figure 6, the fractional disappearance rates of α-T were correlated with plasma ascorbic acid in the cigarette smokers (*P = 0.021, R² = 0.509*) but not in the nonsmokers (*P = 0.503, R² = 0.058*). This observation suggests that the faster fractional disappearance rates of α-T were related to lower ascorbic acid concentrations only in the presence of apparent oxidative stress, as observed in the smokers.

**TABLE 5**

Comparison between total plasma α-tocopherol (α-T) and total urinary α-carboxy-ethyl-hydroxy-chroman (α-CEHC)  

<table>
<thead>
<tr>
<th></th>
<th>α-T (μmol/L)</th>
<th>Total α-T²</th>
<th>α-CEHC (mmol/g creatinine)</th>
<th>Total α-CEHC²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nonsmokers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Smokers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day −6</td>
<td>15.3 ± 2.8</td>
<td>14.6 ± 3.8</td>
<td>540 ± 560</td>
<td>570 ± 834</td>
</tr>
<tr>
<td>Day 0</td>
<td>35.5 ± 5.6</td>
<td>32.1 ± 10.2</td>
<td>3039 ± 2889</td>
<td>2383 ± 1068</td>
</tr>
<tr>
<td>Day 17</td>
<td>19.8 ± 4.0</td>
<td>15.3 ± 3.9</td>
<td>780 ± 842</td>
<td>240 ± 79</td>
</tr>
</tbody>
</table>

1 All values are ̅ ± SD. Supplementation with deuterated (d) α-T resulted in a doubling of total α-T in both the nonsmokers and the smokers (*P < 0.0001*) and in a 5.6- and 4.3-fold increase in total urinary α-CEHC excretion in the nonsmokers and the smokers, respectively.
2 d₆-α- + d₃-α-T.
3 d₆-α- + d₃-α-CEHC.
4 Significantly different from nonsmokers (unpaired Student’s t test): *P = 0.011, †P = 0.039.
Mean uric acid concentrations were observed in either the nonsmokers \((P = 0.076, R^2 = 0.343)\) or the smokers \((P = 0.446, R^2 = 0.074)\).

**DISCUSSION**

Oxidative stress caused by cigarette smoking resulted in increased fractional disappearance rates of \(\alpha\)-T (Figure 4), consistent with an in vivo antioxidant function of \(\alpha\)-T. Smokers and nonsmokers were supplemented with deuterium-labeled \(\alpha\)-T (75 mg each of \(d_3\)-RRR-\(\alpha\)-TAc and \(d_3\)-all-rac-\(\alpha\)-TAc for 6 d (Figure 2). Once supplementation ceased, the fractional disappearance rates and half-lives of \(\alpha\)-T in the smokers were \(\approx 13\% \) greater and \(\approx 10\) h shorter, respectively (Figure 4). As would be expected from these data, the plasma \(d_3\)-\(\alpha\)-T concentrations of the smokers on day 17 were lower than those of the nonsmokers.

In cigarette smokers, but not in nonsmokers, fractional disappearance rates of \(\alpha\)-T were significantly correlated with plasma ascorbic acid concentrations (Figure 6). By multiple linear regression, it was determined that the greatest \(\alpha\)-T utilization occurred in smokers with low plasma ascorbic acid concentration. On the basis of the linear regression analysis of \(\alpha\)-T disappearance rates and mean plasma ascorbic acid concentrations, we estimated that cigarette smokers would require a plasma ascorbic acid concentration of 64.2 \(\mu\)mol/L to have \(\alpha\)-T fractional disappearance kinetics similar to those of the nonsmokers (0.191 pools/d). This calculated estimate is \(\approx 38\%\) higher than the observed mean plasma ascorbic acid concentration of 46.5 \(\mu\)mol/L in the smokers. Because dietary ascorbic acid was linearly correlated with plasma ascorbic acid in the smokers \((P = 0.005, R^2 = 0.697)\), we further calculated that the smokers would require a total dietary ascorbic acid intake of 116 mg to achieve a plasma concentration of 64.2 \(\mu\)mol/L. On average, this estimate would require that smokers consume 43 mg ascorbic acid/d above their reported dietary consumption (Table 3). These calculations are consistent with current dietary ascorbic acid recommendations, which suggest that smokers consume an additional 35 mg ascorbic acid/d above the recommendations for nonsmokers (90 and 75 mg/d for men and women, respectively) (5, 42).

Although fractional disappearance rates of \(\alpha\)-T in smokers were correlated with ascorbic acid concentrations (Figure 6) and smokers and nonsmokers had average plasma ascorbic acid concentrations that were not significantly different (Table 4), it was apparent that the range of ascorbic acid concentrations were greater in the smokers and that low ascorbic acid concentrations were observed in those smokers with the fastest \(\alpha\)-T disappearance rates. These data suggest that both low plasma ascorbic acid concentrations and oxidative stress are necessary to negatively influence the \(\alpha\)-T disappearance kinetics. To our knowledge, this is the first time that this interaction between ascorbic acid and \(\alpha\)-T has been shown in humans. Previously, guinea pigs fed 2 concentrations of deuterium-labeled \(\alpha\)-T and 3 concentrations of ascorbic acid did not show an interaction between these nutrients (43); however, no oxidative stress was applied to the guinea pigs in that investigation. Alternatively, in vitro studies conducted with H4IIE liver cells (44) or erythrocytes (45) subjected to oxidative stress sufficient enough to cause lipid peroxidation showed a sparing effect of \(\alpha\)-T when cells were treated with ascorbic acid. In addition, in another in vitro investigation it was
determined that the \( \alpha \)-tocopheryl radical formed within micellar and bilayer membrane systems could be effectively recycled by ascorbic acid found within the aqueous phase (41).

The results of our investigation showed that smokers have modestly higher requirements of \( \alpha \)-T than do nonsmokers. However, it should not be overlooked that the cohort of participants in this investigation was young (18–25 y) and had a limited history (2–5 y) and frequency (10–20 cigarettes/d) of smoking. Although it cannot be ascertained from the available data in this investigation, we can only speculate that these differences would be greater in older persons with a greater history and frequency of smoking because it is believed that aging in itself is an oxidative stress (46) and, thus, older smokers may require more vitamin E.

We measured plasma isoprostane concentrations as an index of oxidative stress. The smokers had higher plasma isoprostane concentrations than did the nonsmokers throughout the entire study. Although there was a modest reduction in circulating isoprostanes during deuterated \( \alpha \)-T acetate supplementation (days 0–6 to day 0), these changes in isoprostanes were not statistically significant. In our investigation, we did not expect to observe changes in plasma isoprostanes because it has been reported that smokers supplemented with various doses of vitamin E for 5 d (47) or for 3 wk (48) had no significant effect in reducing urinary isoprostane excretion. However, in smokers and in nonsmokers \((n = 100)\) with elevated isoprostanes who were supplemented with 200 mg vitamin E for 1 y, there was a significant reduction in plasma isoprostanes (49). Thus, it is apparent that a longer intervention and possibly a larger sample size might be required to observe a significant reduction in isoprostanes with vitamin E supplementation.

In addition to plasma isoprostanes, we measured plasma FRAP as an index of antioxidant potential. We observed that the smokers, because of their increased oxidative stress, had 12% lower FRAP than did the nonsmokers. These data suggest that smokers have less plasma antioxidant potential, which would be consistent with their greater plasma isoprostane concentrations. Plasma uric acid is the greatest predictor of FRAP and accounts for \( \approx 60\% \) of the total predicted FRAP, whereas ascorbic acid contributes to \( \approx 15\% \) of the value (30). Uric acid has been reported to protect against peroxynitrite-mediated damage in experimental models of multiple sclerosis and central nervous system inflammation (50–52). However, excessive plasma uric acid concentrations are associated with the pathogenesis of gout (53). Both epidemiologic and experimental evidence suggest that uric acid is an independent risk factor for cardiovascular and renal disease (54). Note that all participants in this investigation had uric acid concentrations within normal limits (Table 4), and there were no significant differences in plasma uric acid concentrations between groups throughout the investigation. Therefore, we speculated that the smokers had significantly lower FRAP due to the subtle but nonsignificant differences among some or all of the other predictors of the assay, such as ascorbic acid—which was \( \approx 11\% \) lower \((P > 0.05)\) in the smokers than in the nonsmokers on average—throughout the investigation.

Last, the urinary metabolite of \( \alpha \)-T (\( \alpha \)-CEHC) was lower in the smokers than in the nonsmokers on day 17, which suggests that less \( \alpha \)-T metabolism occurred because there was less excess \( \alpha \)-T available for degradation. In addition, it was calculated that <1% of the labeled \( \alpha \)-T dose was excreted as \( \alpha \)-CEHC in the urine in both the smokers and the nonsmokers. These findings are similar to those of another investigation that analyzed urinary deuterium-labeled \( \alpha \)-CEHC excretion from deuterium-labeled \( \alpha \)-T supplementation (26). Thus, other biological fluids, such as serum (55) or bile and fecal matter (56), may need to be analyzed to estimate the fate of metabolized \( \alpha \)-T.

Collectively, these data suggest that cigarette smokers, because of their elevated oxidative stress, require higher dietary \( \alpha \)-T intakes to maintain plasma \( \alpha \)-T concentrations that are similar to those of nonsmokers. The relation in smokers between faster disappearance rates of \( \alpha \)-T and lower plasma ascorbic acid concentrations warrants further assessment to determine whether intervention with ascorbic acid supplementation can modify \( \alpha \)-T disappearance kinetics in cigarette smokers. In addition, future investigations should attempt to characterize \( \alpha \)-T disappearance kinetics in a larger cohort of smokers with diverse ages and smoking habits, because this investigation had a limited number of young participants who had a relatively short smoking history and a low frequency of smoking.

We thank the subjects for their cooperation and especially thank Scott Leonard (Linus Pauling Institute) for technical assistance with the LC-MS tocopherol analysis. We also thank Wendy McMahan (Montine Laboratory) for performing the plasma isoprostane analysis.

RSB, TMB, and MGT participated in the study design and data collection and analyses and wrote the initial draft of the manuscript. RR conducted the mathematical analysis of \( \alpha \)-T disappearance and participated in the editing and review of the manuscript. TMJ participated in the sample analysis for isoprostanes and contributed to the editing and review of the manuscript. None of the authors had a known conflict of interest.

REFERENCES
ABSTRACT

Background: Phytic acid is a strong inhibitor of iron absorption from fortified foods. In adults, this inhibitory effect can be overcome by adding ascorbic acid with the iron fortificant or by using a “protected” iron compound such as NaFeEDTA. In addition, the use of NaFeEDTA as an iron fortificant has been reported to increase zinc absorption in adult women. No information is available on iron bioavailability from NaFeEDTA or the influence of NaFeEDTA on minerals and trace elements in infants.

Objective: We aimed to compare iron bioavailability from a complementary food based on wheat and soy fortified with either NaFeEDTA or ferrous sulfate plus ascorbic acid. The apparent absorption of zinc, copper, calcium, and magnesium was evaluated in parallel.

Design: Stable-isotope techniques were used in a crossover design to evaluate erythrocyte incorporation of iron 14 d after administration of labeled test meals and the apparent absorption of zinc, copper, calcium, and magnesium was evaluated on the basis of fecal monitoring in 11 infants.

Results: Geometric mean erythrocyte incorporation of iron was 3.7% (NaFeEDTA) and 4.9% (ferrous sulfate plus ascorbic acid) (P = 0.08). No significant differences in the apparent absorption of zinc, copper, calcium, or magnesium were observed between test meals (n = 10).

Conclusions: Iron bioavailability from a high-phytate, cereal-based complementary food fortified with either NaFeEDTA or ferrous sulfate plus ascorbic acid was not significantly different. NaFeEDTA did not influence the apparent absorption of zinc, copper, calcium, or magnesium. NaFeEDTA does not provide any nutritional benefit compared with the combination of a highly bioavailable iron compound and ascorbic acid.

KEY WORDS Iron compound, food fortification, stable isotopes, infant cereal

INTRODUCTION

Iron fortification of complementary foods has long been implemented as a public health strategy in industrialized countries to combat iron deficiency during early life. However, some concern exists about the efficacy and effectiveness of iron-fortified complementary foods for preventing iron deficiency in infants and young children. A major problem related to the potential effect of iron-fortified complementary foods such as infant cereals is that unacceptable organoleptic changes may occur during storage or during food preparation of fortified products containing water-soluble iron compounds with high relative bioavailability. Consequently, non-water-soluble iron compounds are often used to fortify infant cereal products, although some of the most commonly used iron compounds have been shown to have low relative bioavailability and can therefore be expected to have only a limited effect on the iron status of the consumers (1, 2). Furthermore, it is important to note that the bioavailability of iron compounds used in food fortification programs is dependent on the presence of enhancers and inhibitors in the diet. Thus, iron absorption from highly bioavailable iron compounds can be low from products based on cereals and soy because of the presence of phytic acid (3, 4).

Ascorbic acid has been shown to be a potent enhancer of iron absorption in both adults and children (4–10), and this vitamin is therefore often added during the manufacture of industrially produced complementary foods to counteract the inhibitory effect of phytic acid. However, losses of ascorbic acid during processing, storage, and food preparation might limit the usefulness of this approach in some settings. As an alternative to ascorbic acid, Na2EDTA has been evaluated as an enhancer of iron bioavailability in adults and schoolchildren. Although this strategy was shown to be useful to increase iron absorption from ferrous sulfate added to low-bioavailability meals (11–14), no enhancing effect was found when Na2EDTA was added to meals fortified with ferrous fumarate (15, 16).

Clearly, the use of an iron compound with high relative bioavailability whose absorption is not susceptible to the negative effects of inhibitory ligands would be a useful way of providing
iron via fortified foods. NaFeEDTA is a water-soluble iron compound that is less influenced by the presence of phytic acid (13, 15, and reviewed by the International Nutritional Anemia Consultative Group in reference 17). In addition, we reported significantly higher apparent zinc absorption in women, but no effect on calcium absorption, from bread fortified with NaFeEDTA than when ferrous sulfate was used as a food fortificant (18). However, no information is available on iron bioavailability from NaFeEDTA or the influence of NaFeEDTA on minerals and trace elements in infants.

The aim of the present study was to compare iron bioavailability in healthy infants from a complementary food based on wheat and soy and fortified with either NaFeEDTA or an iron compound with high relative bioavailability (ferrous sulfate) under optimal conditions (ie, in the presence of ascorbic acid). Erythrocyte incorporation of iron stable isotopes 14 d after administration was used as a proxy for iron absorption. In parallel, the apparent absorption of zinc, copper, calcium, and magnesium was evaluated by use of a stable-isotope technique based on fecal monitoring.

SUBJECTS AND METHODS

Eleven healthy infants (5 boys and 6 girls, 18–27 wk old) were recruited for the study. Mean birth weight was 3065 g (range: 1885–4055 g). Mean body weight at the time of enrollment into the study was 6992 g (range: 5470–8050 g). All infants were fed primarily cow milk–based infant formula and had been introduced to complementary foods at the time of recruitment. Parents were fully informed about the aims and procedures of the study, and written consent was obtained from at least one parent of each infant. The study protocol was reviewed and approved by the University of Iowa Committee on Research Involving Human Subjects.

The sample size was based on previous data on erythrocyte incorporation of iron stable isotopes in infants (4). It was estimated that 10 infants would be a sufficient sample size to detect a nutritionally significant difference in erythrocyte incorporation of 50% with 90% power and a type I error rate of 5%. Eleven infants were recruited to allow for one dropout. All eleven infants completed the iron absorption study. Ten infants completed the metabolic balance study.

Study design

The study used a balanced, crossover design to evaluate erythrocyte incorporation of iron and the apparent absorption of zinc, copper, calcium, and magnesium from test meals fortified with either NaFeEDTA or ferrous sulfate (FeSO₄). Each study consisted of the ingestion of 2 isotopically labeled test meals followed by the collection of fecal material for 72 h. Capillary blood samples were drawn before and 2 wk after ingestion of the test meals. The second blood sample of the first study was used as the baseline sample for the second study. Test meals were served in a predetermined and random order (NaFeEDTA-FeSO₄ or FeSO₄-NaFeEDTA). Infants were fed 1–2 servings/d of a commercial iron-fortified infant cereal (Ceresoy; Nestlé, Vevey, Switzerland) that was similar to the test meal for 2–3 wk before the start of the study. The prefeeding period was included in the protocol to ensure acceptance of the cereal product by the study infants.

Test meals

An infant cereal based on wheat flour and soy flour was produced especially for this study without any added minerals or vitamins at a Nestlé Product Development Center (Linor, Orbe, Switzerland). Each test meal consisted of 20 g cereal reconstituted with 60 g hot ultrapure water. Labeled test meals were fed after the infants had fasted overnight, or ≥3 h after intake of infant formula, under standardized conditions on day 1 of each study. Stable-isotope labels of iron, zinc, and calcium were added to the first test meal. The second test meal was labeled with stable isotopes of copper and magnesium. The total content of added iron, zinc, and calcium was equilibrated in the second test meal by the addition of minerals with normal isotopic composition. Doses of stable-isotope labels were 888 μg ⁷⁰Zn (ZnCl₂), 1.0 mg ⁶⁵Cu (CuCl₂), 5 mg ²⁵Mg (MgCl₂), and 4 mg ⁴⁴Ca (CaCl₂). Iron was added as 2.0 mg ⁵⁸Fe (FeSO₄) or 2.0 mg ⁵⁸Fe (FeCl₃) mixed with Na₂EDTA as an aqueous solution in a 1:1 molar ratio (Fe:EDTA). Fe:EDTA solutions were prepared immediately before addition to the test meal. Test meals labeled with ⁵⁸FeCl₃ contained added ascorbic acid (l(+)-ascorbic acid; Merck, Darmstadt, Germany) at a molar ratio of iron to ascorbic acid of 1:1.6.

Procedures

Capillary blood samples were drawn before the administration of the first labeled test meal for analysis of hemoglobin and plasma ferritin and for determination of the baseline isotopic composition of whole blood. Body weight and length were recorded. The infants were placed in metabolic beds in the Lora N Thomas Metabolism Ward (Department of Pediatrics, University of Iowa, Iowa City) immediately before intake of the first labeled test meal. The first labeled test meal contained a small amount (50 mg) of carmine red as a fecal marker. A second dose of carmine red was given 72 h after intake of the first dose. Complete collections of fecal material started immediately after intake of the first labeled test meal and continued until the second fecal marker had been excreted as described by Fomon (19). Feces were collected separately from urine in acid-washed heat-resistant glass containers, and special attention was made to avoid contamination during the collection and handling of fecal samples.

During the 72-h balance periods, infants were fed a standardized diet consisting of low-iron infant formula (Similac; Ross, Columbus, OH) fed to satiety and 2 servings each day of 20 g wheat-soy infant cereal. The cereal contained added food-grade FeSO₄ or NaFeEDTA (Dr P Lohman, Emmerthal, Germany) at an iron concentration of 10 mg/100 g cereal product.

Two weeks after intake of the first administration of stable isotopes (day 15), a second capillary blood sample was drawn for measurement of hemoglobin and plasma ferritin and incorporation of ⁵⁸Fe into red blood cells. Body weight was recorded at the same time. The 2 metabolic balances were separated by 2–4 wk. A final blood sample was drawn 2 wk after intake of the second labeled test meal, and body weight was again recorded.

Stable-isotope labels

Highly enriched stable isotopes of ⁷⁰Zn, ⁶⁵Cu, ²⁵Mg, ⁴⁴Ca, and ⁵⁸Fe were purchased from a commercial supplier (Isotec, St-Quentin, France) as metals and were converted into ⁷⁰ZncI₂, ⁶⁵CuCl₂, ²⁵MgCl₂, ⁴⁴CaCl₂, ⁵⁸FeSO₄, and ⁵⁸FeCl₃, respectively.
All solutions were diluted with ultrapure water (18 MΩ; Milli-pore Super Q, Bedford, MA), and individual doses were filled into acid-washed polytetrafluoroethylene vials, flushed with argon, and kept refrigerated until used. Isotopic composition was determined by thermal ionization mass spectrometry [(TIMS) MAT 262; Finnigan MAT, Bremen, Germany].

Sample preparation and analysis

Because of the high risk of contamination during mineral and trace element analysis, special care was taken during sample handling, preparation, and analysis. Only acids purified by sub-boiling distillation and ultrapure water (18 MΩ, Millipore Super Q) were used for the preparation of stable-isotope solutions and for all analytic work. Other chemicals were of analytic grade purity. To minimize contamination through vessel materials, only acid-washed quartz, polytetrafluoroethylene, and polyethylene containers were used. Powder-free gloves were used during all sample handling and analysis.

Fecal material and blood samples were shipped on dry ice to Rüschlikon, Switzerland, for analysis. Fecal material was freeze-dried, ground to a fine powder in acid-washed mortars, and pooled into 72-h pools before further analysis. Fecal pools included the first fecal sample dyed by carmine red and all consecutive stools up until, but not including, fecal material dyed by carmine red. Results from a previous study (20) confirmed that 72 h is a sufficient time period for complete collections of unabsorbed stable isotopes in fecal material in infants consuming infant cereal.

Blood samples

Blood samples were analyzed in duplicate under chemical blank monitoring. Samples of whole blood (0.5 mL) were mineralized in a mixture of 5 mL concentrated HNO3 and 2 mL 30% H2O2 in tetrafluoroethylene-perfluoro (alkoxy vinyl ether)-iron circulating in the body, the amounts of 58Fe label present in blood samples drawn 14 d after test meal administration were calculated. Calculations followed the principles of isotope dilution and considered that the iron stable isotopes were not monoisotopic (26).

Fecal samples

Pooled fecal material was analyzed in duplicate after mineralization in a microwave digestion system (MLS 1200; MLS GmbH) with concentrated HNO3 and H2O2 (30%). Aqueous spikes of 67Zn, 26Mg, and 42Ca were added (prepared as ZnCl2, MgCl2, and CaCl2 solutions in 0.1 mol HCl/L) to determine the amount of natural zinc, magnesium, and calcium in the sample according to isotope-dilution principles. Aliquots were dried and redissolved in 6 mol HCl/L for chromatographic separation of copper and zinc from the matrix. The elemental copper concentration in the fecal material was measured by atomic absorption spectrometry (SpectAA 400; Varian, Mulgrave, Australia) because copper has only 2 stable isotopes and therefore isotope dilution analysis is not an option. Anion-exchange chromatography (AG-1x8, 200–400 mesh; Bio-Rad, Glattbrugg, Switzerland) was used to separate copper and zinc from the matrix by using similar techniques as described earlier (18, 27). The first fraction was kept for further separation of magnesium and calcium. Contamination was monitored during copper separation by the processing of a known amount of pure 65Cu spike in parallel to each batch of samples. The second isotopic label (67Zn added to each sample before sample digestion was used to monitor zinc separation blanks.

The fraction containing magnesium and calcium was evaporated to dryness and re-dissolved in 0.7 mol HCl/L. Cation-exchange chromatography (500WX-8, 200–400 mesh; BioRad) was used to separate magnesium and calcium (27). Separation blanks were monitored by using isotopic labels (26Mg and 42Ca added to each sample before digestion. Zinc, magnesium, and calcium fractions were evaporated to dryness under sub-boiling conditions and were stored in polyethylene vials until analyzed. Copper fractions were evaporated to dryness in capped quartz vessels at 90 °C and were heated at 450 °C for 4 h in a muffle furnace (M110; Heraeus Instruments, Hanau, Germany) to destroy organic matter that might interfere with the isotopic ratio measurements. Isotopic ratios of zinc, magnesium, and calcium were determined for each element by TIMS based on the generation of Zn+, Mg+, and Ca+ ions in a rhenium double-filament ion source similar to a previously described technique (27, 28). A TIMS measurement technique using Cu(CN)2− ions was developed for copper isotopic ratio measurements to improve the precision of isotopic analysis (T Walczyk, personal communication, 2000). Samples (5–10 µg Cu) were loaded as CuCl2 in aqueous solution together with 40 µg Zn as ZnCl2 and 100 µg NaCN on top of the evaporation filament. The solution was dried electrothermally at 0.8 A while the ionization filament remained unloaded. Measurements were performed at ionization filament temperatures of 930 °C and evaporation filament temperatures of ≈350 °C. Ion intensities for the main signal were on the order of 1–2 × 10−11 A.

A single-focusing magnetic sector field TIMS instrument equipped with a multicollector system for simultaneous ion detection (MAT 262; Finnigan MAT) was used for all measurements. At least 50 ion intensity measurements were performed per run by Faraday-Cup detection. Relative reproducibility in isotopic analysis (1 SD) for independent runs of the same sample
was on the order of 0.05% for the $^{65}$Cu/$^{63}$Cu isotopic ratio, 0.1–0.3% for the $^{60}$Zn/$^{64}$Zn and $^{70}$Zn/$^{64}$Zn isotopic ratios, 0.2–0.4% for the $^{25}$Mg/$^{28}$Mg and $^{26}$Mg/$^{28}$Mg isotopic ratios, and 0.2–0.3% for the $^{42}$Ca/$^{43}$Ca and $^{44}$Ca/$^{43}$Ca isotopic ratios, respectively. The shift in isotopic ratios in the fecal material was in the range of 10–20% for copper and 30–50% for zinc, 12–20% for magnesium, and 5–15% for calcium compared with the natural isotopic ratios.

**Calculation of fractional apparent absorption**

The apparent absorption of zinc, copper, magnesium, and calcium was calculated based on the excreted amounts of the $^{70}$Zn, $^{65}$Cu, $^{25}$Mg, and $^{44}$Ca isotopic labels in 72-h fecal pools according to previously described principles (29). Absorbed isotopic labels were determined by subtracting the amount of isotopic labels found in the fecal pools from the administered doses. The total amount of isotopic labels in the fecal pools was calculated from measured isotopic ratios and measured total element amounts. Calculations were based on isotope dilution principles and considered that the isotopic labels were not mono-isotopic (26). Data are presented as fractional absorption of the administered dose. All calculations were performed by using in-house designed software (including control routines) for commercially available spreadsheet software (EXCEL 97, MICROSOFT OFFICE, WINDOWS NT; Microsoft Corporation, Redmond, WA).

**Food analyses**

Samples of infant cereals were mineralized by microwave digestion in a HNO$_3$/H$_2$O$_2$ mixture (MLS 1200; MLS GmbH) and were analyzed for iron, zinc, copper, magnesium, and calcium by electrothermal-flame atomic absorption spectroscopy (SpectRAA 400) by a standard addition technique to minimize matrix effects. Phytic acid content was determined by a HPLC technique (30, 31).

**Statistical evaluation**

Paired t tests (EXCEL 97, MICROSOFT OFFICE, WINDOWS NT; Microsoft Corporation) were used to evaluate differences in erythrocyte incorporation of iron and the apparent absorption of zinc, copper, calcium, and magnesium from test meals fortified with ferrous sulfate and NaFeEDTA. P values <0.05 are referred to as significantly different. Data on erythrocyte incorporation were logarithmically transformed before statistical analysis, and the results are presented as geometric means ±1 SD, −1 SD. All other results are presented as arithmetic means ± SDs.

**RESULTS**

The infant cereal contained 2.0 ± 0.02 mg Fe, 1.1 ± 0.01 mg Zn, 358 ± 4 μg Cu, 55.6 ± 0.9 mg Mg, and 45.0 ± 0.3 mg Ca per 100 g cereal product before the addition of the stable-isotope labels. Corresponding values for the commercial product used during the prefeeding period were 15.1 ± 0.2 mg Fe, 7.3 ± 0.1 mg Zn, 353 ± 6 μg Cu, 45.0 ± 1.0 mg Mg, and 250.6 ± 22 mg Ca. Phytic acid content was 0.41 g per 100 g.

Two infants were anemic (hemoglobin < 110 g/L) and one infant had a low plasma ferritin concentration (<12 μg/L). No significant difference in erythrocyte incorporation of iron stable isotopes was found between the 2 iron fortificants ($P = 0.08$).

Geometric mean erythrocyte incorporation of iron was 3.7% (6.8, 2.0; NaFeEDTA) and 4.9% (10.5, 2.3; ferrous sulfate plus ascorbic acid). Apparent absorption of zinc and copper was 21.1 ± 4.7% compared with 20.5 ± 3.9% ($P = 0.77$) and 11.1 ± 6.2% compared with 8.9 ± 3.0% ($P = 0.24$) from the infant cereal fortified with ferrous sulfate and that fortified with NaFeEDTA, respectively. The corresponding values for calcium and magnesium apparent absorption were 50.0 ± 5.5% compared with 50.6 ± 6.9% ($P = 0.82$) and 49.6 ± 7.4% compared with 47.9 ± 6.1% ($P = 0.53$).

**DISCUSSION**

In the present study, we observed no significant difference in erythrocyte incorporation of iron stable-isotope labels when infants were fed a complementary food based on wheat and soy and fortified with either NaFeEDTA or ferrous sulfate plus ascorbic acid. It is important to stress that ferrous sulfate was evaluated in the presence of ascorbic acid: under these optimal conditions, both iron compounds were equally efficient in providing bioavailable iron from an inhibitory meal.

To our knowledge, the iron bioavailability of NaFeEDTA and ferrous sulfate plus ascorbic acid has not been directly compared previously. In one of our earlier studies, we evaluated the enhancing effect of ascorbic acid and Na$_2$EDTA on iron bioavailability from a cereal-based Peruvian school breakfast meal fortified with ferrous sulfate (14). After the addition of either ascorbic acid or Na$_2$EDTA at molar ratios of 0.6–0.7 relative to iron, no significant difference in iron bioavailability was observed between test meals (14). Thus, both ascorbic acid and Na$_2$EDTA are equally efficient in enhancing iron bioavailability from ferrous sulfate in a Peruvian school breakfast meal. Several studies have reported on the potent enhancing effect of ascorbic acid on iron bioavailability from ferrous sulfate in infants and schoolchildren (4, 9–10, 14). Although the enhancing effect of Na$_2$EDTA on iron bioavailability from inhibitory meals fortified with ferrous sulfate has been shown repeatedly in adults (11–13), there are no other studies in children apart from our study in Peru (14).

The potential usefulness of NaFeEDTA as a fortificant in foods with a high phytic acid content has been shown in several human studies. High relative iron bioavailability from NaFeEDTA added to inhibitory meals, as compared with ferrous sulfate without added ascorbic acid, was shown by MacPhail et al (32), Viteri et al (33), Layrisse & Martinez-Torres (34), Martinez-Torres et al (35), and Davidsson et al (15). However, in less inhibitory meals, iron bioavailability from NaFeEDTA was not significantly different from the iron bioavailability of ferrous sulfate (12, 13, 36). Clearly, the development of a food fortification strategy and, in particular, the selection of an approach to optimize iron bioavailability from the fortified food need careful consideration of the specific conditions relevant to the food fortification vehicle and the target population group. For example, the potential usefulness of NaFeEDTA as a food fortificant for condiments is indicated by the encouraging results from earlier efficacy studies (37–39). There has also been renewed interest in the use of NaFeEDTA as a food fortificant, in particular for iron fortification of liquid condiments, such as fish sauce and soy sauce, because NaFeEDTA can be added without provoking unacceptable organoleptic changes (reviewed by Fidler et al; 36).

In addition, a recent efficacy study in Vietnam provided...
convincing data that fortified fish sauce would have a significant positive effect on the iron status of anemic Vietnamese women (40).

The use of NaFeEDTA as a food fortificant, however, is limited to supervised food fortification programs that provide no more than 0.2 mg Fe · d⁻¹ · kg body wt⁻¹ (41). For infants, the daily intake of iron provided by NaFeEDTA-fortified foods would therefore be limited, and NaFeEDTA is not currently used to fortify industrially produced complementary foods. During the present study, the fortification level was according to that in similar commercial infant cereals (10 mg Fe/100 g dry cereal), and the 2 servings of infant cereal consumed per day provided 4 mg Fe. However, because the infants participating in the present study weighed 5.7–8.0 kg, only 1.2–1.6 mg Fe/d could be provided by NaFeEDTA in order to not exceed the limit of 0.2 mg Fe/kg body wt set by the Joint FAO/WHO Expert Committee on Food Additives (41).

A major reason for the reluctance to use NaFeEDTA in food fortification programs is related to concerns over the possible negative influence of NaFeEDTA on the metabolism of other essential nutrients because EDTA is a strong metal chelator. After digestion, a small fraction (≈ 5%) is absorbed and excreted in urine, whereas the majority is lost via the gastrointestinal tract (42). The absorbed EDTA moiety could negatively affect the metabolism of minerals and trace elements by increased urinary excretion. Only limited information is available on the influence of NaFeEDTA on the absorption and excretion of other nutritionally important minerals and trace elements. We reported on the effect of NaFeEDTA (and of increasing levels of Na₂EDTA) on zinc, copper, and calcium metabolism in rats fed zinc-deficient diets based on soy (43). Although the urinary excretion of zinc increased significantly with the inclusion of EDTA in the diet, the fractional absorption of zinc also increased. Thus, the overall effect was that fractional zinc retention increased significantly in rats consuming diets containing added EDTA. In a later study, we investigated the effects of NaFeEDTA added to high-extraction wheat flour on the absorption and retention of zinc and calcium in adult women by using stable-isotope techniques (18). The results showed a positive effect on zinc apparent absorption from bread fortified with NaFeEDTA compared with ferrous sulfate: mean absorption increased significantly from 20.9% to 33.5% (P < 0.05). Urinary excretion of ⁷⁰Zn increased significantly (P < 0.001) during the 6-d balance period when NaFeEDTA was used as a food fortificant (0.91 ± 0.34%) compared with when bread fortified with ferrous sulfate was consumed (0.29 ± 0.21%). However, zinc retention from the labeled test meals was not significantly different (18).

Our previous observation that NaFeEDTA used as a food fortificant results in increased zinc apparent absorption from inhibitory diets (18, 43) was not confirmed in the present study. Apparent zinc absorption was 21.1 ± 4.7% compared with 20.5 ± 3.9% (P = 0.77) from the cereal fortified with ferrous sulfate or NaFeEDTA in the study infants. Limited information is available on zinc absorption from cereal-based complementary foods in infants. For comparison, we previously reported the mean apparent zinc absorption from a less inhibitory infant cereal (made of wheat flour and cow milk) to be 33.9% (range: 19.2–63.9%) in infants, based on fecal excretion of ⁷⁰Zn (20). No influence on calcium absorption or urinary excretion was observed in the previous studies (18, 43).

In the present study, we evaluated the apparent absorption of both calcium and magnesium from test meals fortified with either NaFeEDTA or ferrous sulfate plus ascorbic acid. Our results support the previous finding that calcium absorption is not influenced by the presence of EDTA in the diet and provide new information on the lack of effect of EDTA on magnesium apparent absorption. We are not aware of any earlier report on the influence of NaFeEDTA on magnesium absorption in humans.

Our previous animal study showed no statistically significant influence on the absorption, excretion, or retention of copper (43). In the present study, copper absorption was 11.1 ± 6.2% and 8.9 ± 3.0% (P = 0.24) from test meals fortified with ferrous sulfate or NaFeEDTA, respectively. No comparable data on copper absorption from cereal products in infants are available. However, we recently reported zinc and copper apparent fractional absorption, based on a stable-isotope technique, from soy formula in 9 healthy infants. In the soy formula study (44), mean zinc and copper absorption were 16.7% and 31.2%, respectively. After dephytinization, absorption of zinc increased significantly (x: 22.6%; P < 0.03), whereas copper absorption was not significantly influenced (x: 35.0%; P = 0.34).

In conclusion, iron bioavailability from a cereal-based complementary food fortified with either NaFeEDTA or ferrous sulfate plus ascorbic acid was not significantly different in healthy infants. NaFeEDTA did not influence the apparent absorption of zinc, copper, calcium, or magnesium. These results indicate that NaFeEDTA does not provide any nutritional benefit compared with the combination of a highly bioavailable iron compound and ascorbic acid. No information is available on the urinary excretion of minerals and trace elements in infants consuming NaFeEDTA. For practical reasons, it was unfortunately not possible in the present study to investigate the urinary excretion of minerals and trace elements. Although this remains a potentially important topic for further studies, the current recommendation by the Joint FAO/WHO Expert Committee on Food Additives of 0.2 mg Fe as NaFeEDTA · d⁻¹ · kg body wt⁻¹ (41) clearly limits the usefulness of this fortificant for infants and children.

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LD designed the study and was responsible for the overall data analysis and the writing of the manuscript. EZ was responsible for the implementation of the study and for data collection. CZ and TW were responsible for the writing of the manuscript. EZ was responsible for the implementation of the study and for data collection. CZ and TW were responsible for the writing of the manuscript. EZ, CZ, and RH contributed to the preparation of the final manuscript. None of the authors had any conflicts of interest.

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Influence of cobalamin deficiency compared with that of cobalamin absorption on serum holo-transcobalamin II

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ABSTRACT

Background: Cobalamin attached to transcobalamin II (TC II), known as holo-TC II, is the active cobalamin fraction taken up by tissues. Holo-TC II is also the form in which absorbed cobalamin enters the circulation from the ileum. Therefore, holo-TC II has been proposed variously as a marker of cobalamin adequacy, cobalamin absorption, or both, including even its advocacy as a surrogate Schilling test. Such claims carry conflicting diagnostic implications because metabolic adequacy and absorption are not identical.

Objective: The objective was to examine metabolic and absorptive influences on holo-TC II.

Design: Treated patients with pernicious anemia (PA), who have abnormal absorption but a normal metabolic status, were chosen as the model to differentiate between the effects of the 2 cobalamin-related characteristics. Serum holo-TC II and indexes of cobalamin metabolism in 23 treated patients were compared with those of 6 untreated PA patients (abnormal absorption and metabolic status) and 33 control subjects (normal absorption and metabolic status).

Results: Holo-TC II, which correlated directly with cobalamin and inversely with homocysteine, was significantly higher in treated PA patients in metabolic remission than in untreated PA patients (74 ± 59 compared with 9 ± 6 pmol/L) and was significantly lower than in control subjects (105 ± 58 pmol/L), although the latter difference was small and the values overlapped greatly.

Conclusions: Metabolic cobalamin status is a major determinant of serum holo-TC II. Absorption status may have mild influence as well, although other explanations remain possible. Serum holo-TC II cannot be used clinically to diagnose cobalamin malabsorption because of overlap with normal values. The influences on holo-TC II are complex and require careful analysis. Am J Clin Nutr 2005; 81:110–4.

KEY WORDS Holo-transcobalamin II, cobalamin, cobalamin absorption, cobalamin deficiency, homocysteine

INTRODUCTION

The diagnostic approach to patients with suspected cobalamin deficiency requires 2 very distinct determinations: the demonstration that deficiency exists and the identification of what caused the deficiency (1). The first step has usually relied on cobalamin assay, but because deficiency is frequently mild or subclinical and because cobalamin concentrations can be falsely low or falsely normal, metabolic tests, such as the measurement of plasma total homocysteine (tHcy), serum or urine methylmalonic acid (MMA), and deoxyuridine suppression, are often done as well (2). Each of these tests has disadvantages, however.

The second step, defining the cause of the deficiency, usually requires ruling malabsorption in or out, with pernicious anemia (PA; ie, the lack of gastric intrinsic factor) as the classical prototype of cobalamin malabsorption. The Schilling test is commonly used, but its availability is declining. Adequate substitutes have not been found; only about half of the cases of PA can be diagnosed through the demonstration of antibodies to intrinsic factor, and no blood test can identify disorders of cobalamin absorption other than PA (1).

Some investigators have advocated the assay of serum holo-transcobalamin II (holo-TC II)—the small, transient fraction of the total cobalamin that is attached to TC II (3). In theory, holo-TC II, the biologically available cobalamin pool in plasma that all cells take up rapidly via specific receptors for TC II (4, 5), seems a more attractive reflection of cobalamin status than does total cobalamin (3), most of which is attached to TC I.

Much of the persisting uncertainty about holo-TC II has both methodologic and conceptual origins (6). Now that accurate assay methods are available (7, 8), the many conceptual uncertainties can be addressed more reliably. One such difficulty was a claim that holo-TC II could serve as a diagnostic index of cobalamin absorption as well as metabolic cobalamin sufficiency (9, 10). Indeed, the holo-TC II concentration was proposed as a “surrogate” for the Schilling test (11). The hypothesis gained currency because TC II binds absorbed cobalamin abuminally in ileal enterocytes and exits into the portal bloodstream (12, 13). Patients with malabsorption might be unable to generate normal amounts of translocated holo-TC II into the bloodstream. However, the quantitative contribution of ileal holo-TC II to plasma holo-TC II is unknown. Moreover, support for serum holo-TC II as a measure of cobalamin absorption is meager, consisting of a study of a few patients with AIDS (9) and of a comparison of holo-TC II with unreliable markers, such as gastric or even duodenal histology, instead of absorption tests (10). Moreover, many studied patients have had deficiency and malabsorption.

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Subjects and Methods

Subjects

Our 2 laboratories, 1 in the United States and 1 in Spain, used their collections of frozen serum (−20 °C) that were left over after clinically indicated diagnostic tests were conducted in patients with confirmed PA. The samples had been collected over a variable time span of several months to 7 y. The key study group was patients with PA whose cobalamin deficiency was treated for ≥2 mo with cyanocobalamin injections, usually after an initial month of weekly or more frequent injections, and were proven to be metabolically normal, although they still had malabsorption.

In all cases, the diagnosis of cobalamin deficiency had been made previously: all patients had megaloblastic anemia, megaloneuropathy, or both and had normal cobalamin concentrations before treatment. The diagnosis of PA, defined as cobalamin malabsorption caused by a loss of gastric intrinsic factor, was established in all patients by one or more of the following tests: a diagnostic Schilling test (low absorption that became normal on retesting with oral intrinsic factor), absence of gastric intrinsic factor in gastric juice collected after pentagastrin stimulation, and presence of antintrinsic factor antibody in a blood specimen obtained at a time remote from cobalamin injection.

The only selection factors were that the clinical and diagnostic information was complete and diagnostically conclusive and that an adequate volume of serum obtained remote in time enough from the previous cobalamin injection was available. The blood was usually obtained just before the next monthly injection, and blood samples with cobalamin concentrations >750 pmol/L were not used. These sampling precautions helped avoid spurious elevation of holo-TC II from the transient saturation of TC II by exogenous cobalamin, which occurs during the first few days after injection. The samples were also assayed for serum cobalamin, plasma tHcy and, if needed, MMA concentrations to ensure that the patients were metabolically cobalamin-replete. Completely normal results from those metabolic tests were obtained in 23 of the 32 serum samples collected from treated patients with PA. In the other 9 samples, however, abnormal metabolic status was identified by a low cobalamin concentration (<180 pmol/L), an elevated tHcy concentration (>14.9 μmol/L in men, and >14.5 μmol/L in women), or an elevated MMA concentration (>279 nmol/L), despite regular monthly cobalamin injections. Holo-TC II concentrations played no role in the metabolic assessment of any patient. To ensure a valid study group of treated patients with PA in complete metabolic remission, the 9 patients were excluded so that their residual biochemical evidence of mild cobalamin deficiency, whatever its precise explanation, would not compromise the interpretation of data in the key study group—the 23 treated patients with PA whose normal metabolic findings satisfactorily indicated cobalamin malabsorption without cobalamin deficiency.

In addition, we tested identically processed and stored samples collected from 6 untreated cobalamin-deficient patients with PA; all 6 had abnormal metabolic findings. Because holo-TC II is known to be low in untreated patients with PA (3, 7, 8), and because holo-TC concentrations in the 6 samples were all low, additional specimens from untreated patients were not sought. The 23 serum samples from patients with treated PA in metabolic remission also included posttreatment samples from 4 of the 6 untreated PA patients; these 4 pairs of matched samples allowed direct comparisons of holo-TC II to be made before and after treatment.

A final group consisted of 33 samples from apparently healthy persons with no clinical or biochemical evidence for cobalamin deficiency. These persons were slightly but not significantly older than the treated PA patients (59 ± 14 compared with 53 ± 17 y). An essential requirement was that the samples be obtained over the same time span from the same institutions and were collected and processed in the same way and stored for durations similar to those for the PA patients’ samples. Although 4 of the 33 control subjects had slightly elevated tHcy concentrations, a not unexpected proportion in normal older people, none of the 4 had abnormal cobalamin or MMA (or holo-TC II) concentrations; similarly, the control subject with a low-normal cobalamin concentration of 189 pmol/L had normal tHcy, MMA, and holo-TC II concentrations. On the basis of cobalamin status, the 33 serum samples thus provided a satisfactory control group for holo-TC II concentrations as well as material for retesting in each assay to confirm the reproducibility of the radioimmunoassay for holo-TC II. The collection of all blood samples met the requirements of our Institutional Review Boards.

Methods

Serum holo-TC II was measured by radioimmunoassay (7), which was performed according to the manufacturer’s protocol that accompanies the kit (Holo-TC; Axis-Shield, Oslo). All sera and standards were assayed in duplicate. To avoid possible influences of timing of magnetic separation among the assay tubes, the duplicate samples were spaced apart from each other in the assay’s pipetting sequence. Serum total cobalamin was measured with the Quantaphase II radioassay (Bio-Rad, Hercules, CA), and plasma tHcy was assayed with the IMx fluorescence polarization immunoassay (Abbott Diagnostics, Abbott Park, IL) in our laboratory. MMA was measured in selected serum samples by gas chromatography–mass spectrometry by Quest Diagnostics (Teterboro, NJ).

Statistical analyses were performed with the assistance of Pavel Kiselev. Analysis of variance was performed by using the general linear model procedure (SAS, version 8.0; SAS Institute, Cary, NC). Because holo-TC II, the main variable of interest, showed a skewed distribution, its values were log transformed to improve the normality characteristics. Analysis of variance was applied with the SAS general linear model procedure to compare log holo-TC II means. Tukey’s method was used to evaluate the
TABLE 1
Serum holo-transcobalamin II (holo-TC II), total cobalamin, and total homocysteine (tHcy) concentrations in all patients with pernicious anemia (PA) and control subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Holo-TC II</th>
<th>Total cobalamin</th>
<th>tHcy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \bar{x} \pm SD )</td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>Control subjects (n = 33)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated PA patients (n = 6)(^2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated PA patients in metabolic remission (n = 23)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated PA patients with residual deficiency (n = 9)(^3)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Mean values in a column with different superscript letters are significantly different, \( P < 0.05 \) (see Methods for statistical analysis procedures).
2 Total cobalamin values were obtained in only 5 of the serum samples because of a limited sample volume.
3 Methylmalonic acid concentrations in these patients ranged from 270 to 504 nmol/L (normal reference <279 nmol/L).

RESULTS

Metabolic comparisons of the groups

As expected, the untreated PA patients had very abnormal serum total cobalamin and tHcy concentrations (Table 1). The differences in cobalamin and tHcy between the untreated patients and the control subjects were significant (\( P < 0.0001 \)). Not surprisingly, the 23 treated PA patients in complete metabolic remission had significantly higher cobalamin concentrations and lower tHcy concentrations than did the untreated patients (\( P < 0.0001 \) for both comparisons), and these concentrations were not significantly different from those of the control subjects (Table 1).

Holo-TC II comparisons of the groups

The distribution of values was skewed in the patients with treated PA in metabolic remission as well as in the control subjects. Both of these groups had significantly higher holo-TC II concentrations than did the untreated PA patients [74 ± 59 and 105 ± 58 pmol/L, respectively, compared with 9 ± 6 pmol/L, \( P < 0.0001 \) (ANOVA), which were significant at the 0.01 concentration using Tukey’s method; Table 1]. Holo-TC II concentrations in the treated PA patients in metabolic remission were also lower than those in the control subjects (\( P = 0.0045 \), which was significant at the 0.05 level using Tukey’s method). Although the difference was significant, there was nevertheless great overlap of the treated PA values with the control values, which did not exist in comparison with untreated PA patients. Values for the treated PA patients and for the control subjects could not be clearly separated. Moreover, none of the holo-TC II concentrations in the treated PA patients in remission were <30 pmol/L, which placed them all within the published reference interval for the holo-TC II assay (7) and contrasted sharply with the values in the untreated patients with PA.

Not surprisingly, holo-TC II results intermediate between those of the treated PA patients in metabolic remission and the untreated PA patients were seen in the 9 patients with treated PA whose cobalamin deficiency, because of incomplete treatment, incomplete responses, or other reasons, persisted despite treatment (Table 1), as shown by persistently low cobalamin, elevated tHcy, or elevated MMA concentrations or a combination thereof.

Direct comparisons of holo-TC II before and after treatment

Holo-TC II findings in 4 of the 6 patients with treated PA in remission could also be compared directly with their own pretreatment concentrations (Table 2). The initially low holo-TC II concentrations (1–9 pmol/L) became completely normal in all 4 patients (57–130 pmol/L) when measured in sera obtained at a time remote from the last previous cobalamin injection.

Correlations of holo-TC II concentrations with other biochemical markers

Serum holo-TCII concentrations correlated directly with total cobalamin concentrations (\( r = 0.45, P = 0.0001 \)) and inversely with tHcy concentrations (\( r = −0.41, P = 0.001 \)).

DISCUSSION

Circulating concentrations of holo-TC II presumably mirror a balance between cobalamin availability and status, holo-TC II
elaboration and release from the gut and other sources, and up-
take by TC II-receptor-mediated endocytosis. The diagnostic
ramifications of serum holo-TC II are controversial, in part be-
cause the details of holo-TC II regulation and influences on it are
still uncertain and probably vary. Practical issues being debated
are whether holo-TC II assay provides any diagnostic advantages
over the measurement of serum total cobalamin, whether de-
creased holo-TC II represents the earliest sign of cobalamin
depletion, whether less central cobalamin-related phenomena,
such as malabsorption, affect serum holo-TC II concentrations,
and whether phenomena entirely unrelated to cobalamin status
also do so.

The questions are complicated by the variety of possible
sources of circulating holo-TC II and what determines its clear-
ance (6). Besides the gut, which provides holo-TC II carrying
both ingested and enterohepatically recycled cobalamin (12, 13),
the kidney (14), and perhaps other tissues (eg, the liver) may also
contribute a continuous stream of holo-TC II. Kidney and liver
probably play important roles in clearance as well (14–16), and
cobalamin-unrelated changes such as diseases of these or other
tissues may affect holo-TC II homeostasis. For example, kidney
and liver diseases are associated with elevated holo-TC II con-
centrations (17, 18), and decreased holo-TC II has been attributed
to increased uptake by erythroid hyperplasia of the bone marrow
(19). Indeed, low holo-TC II concentrations often have been
noted in patients without cobalamin deficiency (10, 19). Some-
times, the tendency exists to explain isolated low holo-TC II
concentrations as the earliest marker of cobalamin depletion, one
that is even more sensitive than is tHcy or MMA. However, such
claims are inherently unprovable, especially when so little is
known about alternative explanations for low holo-TC II con-
centrations. The need for study of these unresolved questions
with the improved assays now available led us to examine the
effect, if any, of malabsorption on holo-TC II.

Our data confirmed the major influence of cobalamin status on
holo-TC II, which was greatly decreased in untreated PA patients
with cobalamin deficiency and became normal (>30 pmol/L)
once the deficiency was corrected. The highly significant rela-
tion with metabolic status despite the persistence of malab-
sorption indicates that circulating holo-TC II reflects cobalamin
metabolic status more closely than it does absorption status.
Nevertheless, holo-TC II concentrations were also significantly
lower in the patients with treated PA in metabolic remission than
in healthy control subjects. The difference was small, but the data
suggest that cobalamin absorption modifies serum holo-TC II
concentrations to some extent, creating a subtle, complex inter-
play of influences. Alternative explanations for the tendency for
lower concentrations in these patients than in control subjects, oth-
er than the effect of malabsorption, are nevertheless possible.
These explanations include incompletely repleted cobalamin
stores despite restored metabolic function, increased holo-TC II
turnover due to an increased demand for cobalamin or to altered
enterohepatic recirculation in PA regardless of treatment, and the
possibility that some of our patients had residual metabolic de-
ficiency that was undetected by current tests.

Despite the apparent influence of malabsorption on serum
holo-TC II, the effect was not as great as was that of poor met-
abonomic status, which produces markedly subnormal holo-TC II
concentrations. No concentrations outside the normal range were
apparent in the treated PA patients in metabolic remission, and
overlap with control values was great. These findings, especially
the overlap, rule out a practical diagnostic use for serum holo-TC
II as an index of cobalamin absorption status and, certainly, as a
surrogate Schilling test. Past interpretations of holo-TC II find-
ings and conclusions based on such assumptions require reass-
essment.

A new and somewhat related diagnostic question must also be
raised. The now apparent, albeit small, influence of absorption
may compromise the frequently proposed role of holo-TC II as a
reliable measurement of metabolic cobalamin status rivaling that
of MMA or tHcy. The case made for low holo-TC II concentra-
tions as the earliest marker of cobalamin deficiency owes much
to studies of variably cobalamin-deficient patients who often also
had PA and other forms of cobalamin malabsorption (20), in-
cluding food-cobalamin malabsorption which is much more
common than PA (21). It must now be reconsidered whether the
mild early holo-TC II decreases observed in some studies may
have reflected malabsorption rather than the earliest sign of met-
abolic deficiency.

Several potential limitations of our study merit comment. The
number of patients, especially those with untreated PA, was
relatively small because PA is uncommon and collecting highly
selected samples from well-characterized patients is difficult.
Nevertheless, the sample size even for the untreated patients
sufficed in providing clear data resembling those reported in the
literature and providing statistically significant findings. The
patients with untreated PA were also not central to our hypo-
thesis, which rested on treated PA patients. Another potential lim-
itation was that many of the serum samples had been stored at
−20 °C for periods of up to several years. The effect of such
storage has not been well studied and will require retesting sam-
ple over several years. We addressed this problem in the study
by selecting control sera that were subjected to storage and han-
dling conditions that were nearly identical to those for the PA
sera, thus presumably neutralizing potential but currently un-
known storage and handling effects in the PA sera. Moreover, our
results in control subjects and in PA patients agreed with those in
the fresher samples in our laboratory as well as with existing data
in the literature. Finally, it is clear that cobalamin injection has
immediate, direct, but transient effects on serum holo-TC II (and
total cobalamin) that can distort interpretation. No data exist on
the duration and arc of this artifactual inflation of holo-TC II or
the optimum time for sampling, which probably vary among
individuals. However, given the measured half-life of only a few
hours for holo-TC II (22), the therapy-induced artifact is proba-
bly dissipated after several days and almost certainly by 1 or 2
weeks. Sampling in our study was done several weeks after the
last previous cobalamin injection to minimize the risk of such an
artifact. For further assurance, sera with elevated total cobalamin
concentrations were excluded from study to further avoid the risk
of transient injection-induced holo-TC II artifacts.

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statistical assistance.

XC and RC were involved in the design of the study, the data collection
and analysis, and the writing of the manuscript. AFR was involved in the data
collection and analysis and provided advice. MPS was involved in the data
collection and analysis. None of the authors had a financial interest or other
conflicts of interest related to this work.
REFERENCES


Iron deficiency due to consumption of a habitual diet low in bioavailable iron: a longitudinal cohort study in Moroccan children

Michael B Zimmermann, Nourredine Chaouki, and Richard F Hurrell

ABSTRACT

Background: In many developing countries, cereal and legume-based diets contain low amounts of bioavailable iron, which may increase the risk of iron deficiency.

Objective: The objective was to measure change in iron status in Moroccan children who consumed their habitual diet containing low amounts of bioavailable iron.

Design: The design was a prospective, longitudinal, free-living cohort study in iron-replete, nonanemic 6–10-y-old children (n = 126). Hemoglobin, serum ferritin, and transferrin receptor were measured at baseline. The children then consumed their habitual cereal and legume-based diet for 15 mo, when their iron status was retested. We used weighed food records and direct food analysis to calculate dietary iron intake and iron bioavailability. On the basis of the change in hemoglobin and body iron stores calculated from the serum transferrin receptor–to–ferritin ratio, iron balance and iron absorption were estimated over the 15-mo period.

Results: Mean daily iron intake was 10.8 mg/d, 97% of which was nonheme iron. Estimated nonheme-iron bioavailability from algorithms was 1.0–4.3% adjusted for low body iron stores. Over 15 mo, the mean change in total body iron was −142 mg, and mean iron absorption was estimated to be 0.22 mg/d, or 2% of dietary iron. Mean hemoglobin concentration decreased 12 g/L. At 15 mo, 75% of the cohort had deficits in tissue iron, and one-third had mild iron deficiency anemia.


KEY WORDS Iron deficiency, bioavailability, diet, anemia, children, Morocco

INTRODUCTION

Iron deficiency anemia (IDA) is common among children in developing countries, where the prevalence is often 50% or more (1). Iron balance in childhood is maintained by adjusting the rate of iron absorption to meet the increased needs for growth and expansion of the red blood cell mass and to cover basal losses from the skin and genitourinary and gastrointestinal tracts. During the first decade of life, daily needs for absorbed iron in children increase from 0.5 to 0.8 mg (2), a relatively high requirement given their smaller body size and food intake. In many developing countries, monotonous cereal and legume-based diets contain low amounts of bioavailable iron. These diets often contain little meat, supply mainly nonheme iron, and are high in inhibitors of nonheme-iron absorption (eg, phytic acid) and low in enhancers of absorption (eg, animal tissue and ascorbic acid) (3).

Although low iron bioavailability is thought to play a central role in the etiology of IDA in developing countries (1, 3), little direct scientific evidence supports this claim. Epidemiologic associations between serum ferritin (SF), anemia, or both and dietary components, such as animal tissue and ascorbic acid, suggest that iron bioavailability could influence iron stores (4, 5). However, correlations in most cross-sectional studies were modest, and several studies found no correlation (6, 7). Although iron bioavailability strongly influences nonheme-iron absorption from single meals (3), longitudinal studies lasting weeks or months indicate little or no response of body iron stores (estimated from SF) to changes in dietary iron bioavailability, including changes in intakes of ascorbic acid (8, 9) and meat (10). This disparity may be at least partly explained by long-term adaptation in iron absorption to maintain iron stores (11). Children in developing countries with low iron stores may be able to regulate iron absorption from cereal-based diets to preserve hemoglobin mass. It was argued that vitamin A deficiency or blood loss from parasitic infections, rather than iron bioavailability, are important causes of IDA (7, 12, 13).

In rural northern Morocco, the prevalence of IDA in school-age children is ≈35% (14). We recently had the opportunity to follow up a cohort of rural Moroccan school children who had been made iron replete by their participation in a successful efficacy trial of iron fortification (14). When that trial ended, the children resumed their customary cereal and legume-based diet at home containing no fortification iron. We measured their dietary intakes by using weighed food records and tested their iron status 15 mo later, at the beginning of a second iron fortification trial. Our aim was to determine the effects of a diet of low iron bioavailability on iron status in the cohort.
SUBJECTS AND METHODS

Study site

The study was done in a cluster of rural villages in the Brikcha Rural Commune, in the Rif Mountains of northern Morocco. The villages are 500–700 m above sea level and have a temperate climate, with an 8-mo dry season (22–34 °C; mean rainfall: 23 cm/mo), and 4-mo damp season (10–22 °C; mean rainfall: 77 cm/mo). The villages comprise ≈450 households, with a total population of ≈3600 individuals of mixed Berber and Arab descent. The villages are isolated from commercial routes, being ≈5–10 km from the nearest through paved road. Approximately one-half of the village households do not have electricity or running water. Agriculture employs >95% of the working population, and most food consumed is produced locally on small farms (15). The main foods grown are wheat, barley, dry legumes (fava beans, chickpeas, lentils), and olives. Cereal plantings occupy 45–50% of the area suitable for agriculture, dry legumes 15–20%, with the remainder being mainly olive trees (15). There is a small amount of livestock, mainly goats destined for milk and meat production.

Measurement of dietary iron intake and estimation of iron bioavailability

To determine food intake in the villages, 3-d weighed food records were done in 50 households randomly selected from local census rolls. The records were done by 3 trained university graduates born in the villages and fluent in the local Arab dialect. They knew the families they were surveying personally and were familiar with local food customs. Households were asked to maintain their usual food habits and their traditional ways of cooking and serving foods. To account for potential seasonal variations in the diet, 24 households were studied in the winter and 26 during the summer. The records were done under the direct supervision of an experienced member of the research team on the first day of the 3-d survey in each family.

Over 3 consecutive days, the surveyors weighed the edible portions of all food and beverages consumed by the families with use of a Kern 440-53 scale (D-72458; Kern & Sohn GmbH, Albstadt, Germany) accurate to 1 g and calibrated with use of fixed weights each week during the study. All uneaten leftovers (mainly skin and bones from meat and stones from fruit) were weighed separately and subtracted. The age and sex of the individuals participating in each meal were recorded. Local food habits on Friday differed from the remaining days of the week. On Friday either a large couscous or a tagine are consumed, and both dishes are rich in meat and vegetables. Therefore, records were kept in half of the families on 2 weekdays and a Friday and in the other half on 3 days not including a Friday. At the end of each day, members of the household were asked if a meal or snack was consumed outside the home (this was rare). If so, its content was estimated and recorded. The completeness and coding of the records were checked by the supervisor at the end of each day.

The iron and phytic acid content of 24 local foods that formed an important part of the diet were analyzed in Zürich. These foods included legumes (lentils, chickpeas, fava beans, and white beans), olives, vegetables, cereals (whole and refined wheat flour, semolina, potato, rice). Freeze-dried food samples (500 mg) were hydrolyzed by microwave digestion (MLS 1200; Microwave Laboratory Systems GmbH, Leutkirch im Allgäu, Germany), and the iron concentration was measured by atomic absorption spectrometry with use of a graphite furnace (SpectraAA-300/400 with GTA-96 Graphite Tube Atomizer; Varian Techtron Pty Ltd, Mulgrave, Victoria, Australia). Phytic acid in foods was measured by using the modified Makower method (16). Triplicate portions of all food samples were analyzed and values were averaged. Analyzed values for iron (>95% of the total iron in the diet came from the 24 foods that were analyzed) and phytic acid were used with data on other nutrients from the Moroccan Food Composition Table (17) and, when necessary, the Food and Agriculture Table for Africa (18).

In this region, meals consist of 1 or 2 communal dishes placed in the center of the table from which all family members eat with their hands. We therefore estimated individual food consumption by using the unit of consumption (UC) formula used by the Department of Agriculture of Morocco (15). That is for each male older than 14 y, UC = 1.0; for each female older than 10 y, UC = 0.8; and for each male aged 14 y or younger and each female aged 10 y or younger, UC = 0.3 + [0.05 × age (in y)]. To determine individual nutrient intakes for a meal, the overall sum of a nutrient for a family was divided by the total amount of UCs participating in that meal. Vitamin A intakes were calculated as retinol activity equivalents (RAEs), using a conversion factor of 12 μg β-carotene to 1 μg retinol (19).

The algorithms of Tseng at al (20) and Reddy at al (21) were used to estimate iron bioavailability. It was assumed that only meat, fish, and poultry (MFP) contained heme iron. In meat, poultry, and fish, 50%, 40%, and 20% of iron was estimated to be heme iron, respectively, with the remainder being nonheme iron. To estimate nonheme-iron absorption for a range of body iron, results of the algorithms were adjusted for high, medium, and low body iron stores (22). It was assumed that the absorption of heme iron was stable in the presence of either enhancers or inhibitors at a rate of 23% for high iron stores, 28% for medium stores, and 35% for low stores (23).

Cohort study

The baseline measurements for this study were done at the completion of a 1-y iron fortification trial in primary school children in the villages (14). In that trial, the addition of encapsulated ferrous sulfate to salt was highly efficacious in reducing the prevalence of IDA. The cohort for the present study was enrolled from that study population by including all 6–10-y-old children who 1) were nonanemic (hemoglobin ≥ 115 g/L), 2) were iron sufficient [SF ≥ 15 μg/L and transferrin receptor (TIR) ≤8.5 mg/L], 3) had a serum C-reactive protein (CRP) concentration <10 mg/L (to reduce the confounding effect of inflammation on SF), and 4) were menstruating. One hundred thirty-four children met these inclusion criteria. When distribution of the iron-fortified salt stopped at completion of the efficacy trial in December 2001, the children resumed their habitual diets at home containing no fortification iron.

In April 2003, in preparation for a second set of iron fortification trials in the area, all children in the same 3 primary schools underwent another screening, with measurements of height, weight, hemoglobin, SF, TIR, serum retinol (SR), and CRP. Of the 134 children who were originally enrolled, 4 had moved away, 2 declined venipuncture, 1 had an elevated CRP, and 1 had begun menstruating. These 8 children were excluded, leaving a cohort of 126 children with complete measurements at baseline and 15 mo later. Informed written consent (or, if parents were
illiterate, oral consent) was obtained from the parents and oral assent from the children. The Swiss Federal Institute of Technology Zürich and the Ministry of Health in Rabat gave ethical approval for the studies. After the 15-mo measurements were collected, the cohort in this study joined the remainder of the children in the schools in a new iron fortification trial, which started in May 2003.

Blood from venipuncture was collected into tubes containing EDTA and transported on ice to the local laboratory. Hemoglobin was measured in whole blood (refrigerated and measured on the day of collection) with use of an AcT8 Counter (Beckman Coulter, Krefeld, Germany), using controls provided by the manufacturer. Anemia was defined as a hemoglobin concentration <115 g/L in children aged 6–11 y (1). Serum samples were divided into aliquots and frozen at −20 °C until analysis. SF and TfR were measured by using enzyme-linked immunosorbent assays (RAMCO, Houston, TX), with controls provided by the manufacturer. For SF, at concentrations of 17 and 94 μg/L, interassay CV was 24.4% and 11.2%, and intraassay CV was 9.2% and 9.1%. For TfR, at concentrations of 5.9 and 15.9 mg/L, interassay CV was 6.4% and 10.0%, and intraassay CV was 6.6% and 10.1%. Iron deficiency was defined as either SF <15 μg/L or TfR >8.5 mg/L. CRP was measured by using nephelometry (TURBOX; Orion Diagnostica, Espoo, Finland); values >10 mg/L were considered elevated. SR was measured by HPLC, and vitamin A deficiency was defined as a SR <0.70 μmol/L.

Data analysis

Within-subject and between-subject coefficients of variation in daily iron intake were calculated and used to determine the precision of our estimate of the mean daily iron intake for the cohort, using the method of Beaton et al (24), as shown in Equation 1:

\[ D_t = Z_a \times CV_b g / CV_w g n \]

where \( D_t \) is the greatest deviation from the mean as a percentage of long-term true intake (half of the 95% CI of the mean), \( Z_a \) is the normal deviation for the percentage of times the measured value should be within a specified limit (1.96 for 95% confidence), \( CV_b \) is the between-subject CV, \( CV_w \) is the within-subject CV, \( g \) is the number of subjects, and \( n \) is the number of days measured.

The 4 principal components of the iron requirement in young children are basal iron losses, increase in storage iron, increase in nonstorage iron in tissues, and increase in hemoglobin iron (2). Each of these components was calculated for the individual children in the cohort.

**Basal iron losses**

Daily basal losses of iron were calculated as follows (25, 26):

**Basal iron losses** = \( 0.538 \times \text{body surface area (BSA; in m}^2) \)

(2)

where

\[ \text{BSA(m}^2) = \text{weight (kg)}^{0.538} \times \text{height (cm)}^{0.3964} \times 0.024265 \]

(3)

For the calculation of BSA, the mean of the weights and heights from the baseline and 15-mo measurements were used.

**Body iron stores**

Body iron stores were estimated from the ratio of serum TfR to SF as follows (27):

**Body iron (mg/kg)** = \( -[\log(TfR/SF \text{ ratio}) - 2.8229]/0.1207 \)

(4)

Positive values indicate the amount of iron in stores, and negative values indicate the deficit in tissue iron.

**Nonstorage iron in tissues**

It was assumed nonstorage iron in tissues, mainly in enzymes essential for cell function, would be conserved despite overall negative iron balance. Nonstorage iron was calculated as follows (28):

**Nonstorage iron** = \( 0.7\text{mg[body weight (kg) at 15 mo - body weight (kg) at baseline]} \)

(5)

**Iron in hemoglobin**

Blood volume (BV) was calculated from weight and height at baseline and at 15 mo as follows (29):

For boys: \( \log \text{BV (mL)} = (0.6459) \log \text{weight (kg)} + (0.002743) \text{height (cm)} + 2.0324 \)

(6)

For girls: \( \log \text{BV (mL)} = (0.6412) \log \text{weight (kg)} + (0.001270) \text{height (cm)} + 2.2169 \)

(7)

The change in hemoglobin (Hb) mass (in g) was calculated as follows:

**ΔHb mass** = \( B[\text{Hb (L) × Hb concentration (g/L) at 15 mo] - [\text{Hb (L) × Hb concentration (g/L) at baseline}] \)

(8)

The change in iron in Hb was then calculated as follows (2):

**ΔHb iron (mg)** = \( Δ\text{Hb mass (g)} × 3.39 \text{mg Fe/g Hb} \)

(9)

The local health records of the past 2 y were reviewed with the chief medical officer. There were no cases of malaria, and treatment of infections with *Necator americanus*, *Trichocephalus trichiura*, or *Schistosoma mansoni* and *Schistosoma hematobium* were rare and confined to individuals with a history of recent travel to endemic areas. Treatment of infections with *Anklylostoma duodenale*, often found in the Mediterranean basin, was uncommon. Because of the dry, temperate climate and clean public water supply, parasites that cause blood loss are rare in children in northern Morocco. We therefore assumed that there were negligible iron losses in the cohort other than obligatory basal losses.

The final balance equation used to estimate the amount of dietary iron absorbed over the 15-mo study was as follows:

**Dietary iron absorbed** = **basal iron losses** + **nonstorage iron in tissues** + **ΔHb iron** + **Δbody iron stores** (10)
from 0 to 15 mo as the dependent variable, and change in height, calculated with the change in body iron (mg/kg body weight) multiple comparisons. Stepwise linear regression models were transformed before analysis. Data at baseline and at 15 mo were logarithmically transformed before analysis. Data at baseline and at 15 mo were compared by using paired t tests with a Bonferroni correction for multiple comparisons. Stepwise linear regression models were calculated with the change in body iron (mg/kg body weight) from 0 to 15 mo as the dependent variable, and change in height, energy; the main source was fish (68% of MFP, mainly sardines). Total dietary iron (mg/d) of this was from olives, olive oil, or both consumed at nearly every meal. Legumes contributed 4% of total energy and included mainly fava beans along with chickpeas and lentils. MFP contributed 5% of dietary energy; the main source was fish (68% of MFP, mainly sardines).

### Statistical analysis

Data processing and statistics were done with use of PRISM3 (GraphPad, San Diego, CA) and EXCEL 97 (Microsoft, Seattle, WA). Variables not normally distributed were logarithmically transformed before analysis. Data at baseline and at 15 mo were compared by using paired t tests with a Bonferroni correction for multiple comparisons. Stepwise linear regression models were calculated with the change in body iron (mg/kg body weight) from 0 to 15 mo as the dependent variable, and change in height, energy; the main source was fish (68% of MFP, mainly sardines). Total dietary iron (mg/d) of this was from olives, olive oil, or both consumed at nearly every meal. Legumes contributed 4% of total energy and included mainly fava beans along with chickpeas and lentils. MFP contributed 5% of dietary energy; the main source was fish (68% of MFP, mainly sardines).

### RESULTS

#### Weighed food records

Fifty families and a total of 322 subjects (median age: 19 y; range: 2–74 y) participated in the 3-d weighed food records. Sixty-three subjects were 6–10-y-old children. The dietary staple was wheat flour made up of bread that was consumed at every meal. Approximately 45% was whole-wheat flour and 55% was refined wheat flour (“farine de luxe”). Overall, cereals contributed 58% of the total energy in the diet. Eighteen percent of total energy came from oil and fats; >90% of this was from olives, olive oil, or both consumed at nearly every meal. Legumes contributed 4% of total energy and included mainly fava beans along with chickpeas and lentils. MFP contributed 5% of dietary energy; the main source was fish (68% of MFP, mainly sardines).

#### Vegetables

Vegetables consisted of mainly potatoes, tomatoes, and onions and supplied <3% of total energy.

Daily mean (± SD) energy and protein intakes in 6–10-y-old children were 200 ± 273 kcal, and 27 ± 6 g, respectively. Mean (± SD) vitamin A intake was 247 ± 69 RAE/d, with 69% as retinol and 31% as carotenoids from plant foods. By direct analysis, the iron and phytic acid contents (mg/100 g dry weight) of the principal staples were the following: whole wheat flour, 1.91 ± 0.09 mg iron and 358 ± 35 mg phytic acid; refined wheat flour, 1.58 ± 0.03 mg iron and 83 ± 5 mg phytic acid; lentils, 6.87 ± 0.90 mg iron and 589 ± 28 mg phytic acid; fava beans, 4.15 ± 0.56 mg iron and 595 ± 25 mg phytic acid; and chickpeas, 6.34 ± 0.43 mg iron and 823 ± 21 mg phytic acid.

### Cohort study

The changes in the cohort over the 15-mo study are shown in Table 2. Mean (± SD) iron intake was 10.8 ± 2.3 mg/d, 97% of which was nonheme iron. Cereals (mainly wheat flour in bread) contributed >90% of dietary phytic acid. Vegetables contributed 81% of dietary ascorbic acid. Comparing winter with summer, there were no significant differences in within-subject CV or between-subject CV in daily iron intakes and no significant differences in mean daily intakes of iron, phytic acid, or ascorbic acid (data not shown). Overall, the within-subject CV in daily iron intake was 17.1%, and the between-subject CV in daily iron intake was 21.3%. Therefore, the precision of the estimated mean daily iron intake in the cohort from the 3-d weighed food records was adequate; the 95% CI of the group mean was ±5.8%. Because the diet was high in phytic acid and the molar ratio of ascorbic acid to iron was low (=1:1), estimated nonheme-iron bioavailability was only 0.3 – 1.3% and 1.0 – 4.3% for high and low body iron stores, respectively (Table 1). Calculated total dietary iron absorbed daily in 6–10-y-old children assuming high, medium, and low body iron stores is shown in Table 2.

### Cohort study

The changes in the cohort over the 15-mo study are shown in Table 3. Mean weight and height increased by 2.0 kg and 5.0 cm, respectively (P < 0.05). Although mean hemoglobin mass decreased only 2.5%, blood volume expanded 7.5% (P < 0.05), so that mean hemoglobin concentration fell 12 g/L (P < 0.01). This

### Table 1

Dietary intakes of iron and enhancers or inhibitors of iron absorption in 6–10-y-old children consuming their customary diet in rural northern Morocco

<table>
<thead>
<tr>
<th>Intake per 1000 kcal</th>
<th>Intake per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total dietary iron (mg)</td>
<td>5.7 ± 1.1</td>
</tr>
<tr>
<td>Nonheme iron (mg)</td>
<td>5.2 ± 1.1</td>
</tr>
<tr>
<td>Heme iron (mg)</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>Phytic acid (mg)</td>
<td>846 ± 89</td>
</tr>
<tr>
<td>Ascorbic acid (mg)</td>
<td>19.6 ± 11.2</td>
</tr>
<tr>
<td>Meat, fish, and poultry (g)</td>
<td>27.7 ± 16.2</td>
</tr>
</tbody>
</table>

### Table 2

Estimated iron absorption in 6–10-y-old children consuming their customary diet in rural northern Morocco, adjusted for high, medium, and low iron stores

<table>
<thead>
<tr>
<th>Percentage nonheme iron absorption (%)</th>
<th>High</th>
<th>Medium</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algorithm of Reddy et al (21)</td>
<td>0.3 ± 0.2</td>
<td>0.6 ± 0.4</td>
<td>1.0 ± 0.8</td>
</tr>
<tr>
<td>Algorithm of Tseng et al (20)</td>
<td>1.3 ± 0.3</td>
<td>2.9 ± 0.6</td>
<td>4.3 ± 0.9</td>
</tr>
<tr>
<td>Total absorbed dietary iron (mg/d)</td>
<td>0.10 ± 0.01</td>
<td>0.14 ± 0.02</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>Algorithm of Reddy et al (21)</td>
<td>0.20 ± 0.05</td>
<td>0.38 ± 0.07</td>
<td>0.55 ± 0.11</td>
</tr>
<tr>
<td>Algorithm of Tseng et al (20)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 All values are x ± SD; n = 63.
2 From Cook et al (22): high stores, serum ferritin concentrations of 65 µg/L; medium stores, serum ferritin concentrations of 30 µg/L; low stores, serum ferritin concentrations of 20 µg/L.
3, 4 Significantly different from high stores (paired t tests with Bonferroni correction): 3P < 0.05, 4P < 0.01.
5, 6 Significantly different from high stores (paired t tests with Bonferroni correction): 5P < 0.001, 6P < 0.001.
TABLE 3
Anthropometric, biochemical, and hematologic variables in a cohort of 6–10-yr-old Moroccan children who were nonanemic and iron-sufficient at baseline and consumed their habitual diet (low in bioavailable iron) for 15 mo

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>15 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>8.2 ± 1.1</td>
<td>9.4 ± 1.1</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>129 ± 8</td>
<td>134 ± 8</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>26.3 ± 3.7</td>
<td>28.3 ± 3.9</td>
</tr>
<tr>
<td>Blood volume (L)</td>
<td>1.99 ± 0.24</td>
<td>2.14 ± 0.26</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>128 ± 9</td>
<td>116 ± 9</td>
</tr>
<tr>
<td>Hemoglobin mass (g)</td>
<td>255 ± 38</td>
<td>248 ± 38</td>
</tr>
<tr>
<td>Hemoglobin iron (mg)</td>
<td>863 ± 129</td>
<td>841 ± 128</td>
</tr>
<tr>
<td>Serum ferritin (µg/L)</td>
<td>34 ± 14</td>
<td>13 ± 12</td>
</tr>
<tr>
<td>Transferrin receptor (µg/L)</td>
<td>6.9 ± 1.1</td>
<td>9.7 ± 4.5</td>
</tr>
<tr>
<td>Body iron stores (mg/kg)</td>
<td>4.1 ± 1.5</td>
<td>−1.1 ± 3.5</td>
</tr>
<tr>
<td>Total body iron stores (mg)</td>
<td>106 ± 40</td>
<td>−15 ± 76</td>
</tr>
<tr>
<td>No. of children with anemia (%)</td>
<td>0</td>
<td>54 [43]</td>
</tr>
<tr>
<td>No. of children with iron deficiency anemia (%)</td>
<td>0</td>
<td>42 [33]</td>
</tr>
<tr>
<td>No. of children with tissue iron deficit (%)</td>
<td>0</td>
<td>95 [75]</td>
</tr>
<tr>
<td>Serum retinol (µmol/L)</td>
<td>—</td>
<td>0.92 ± 0.15</td>
</tr>
<tr>
<td>No. of children with vitamin A deficiency (%)</td>
<td>—</td>
<td>15 [12]</td>
</tr>
</tbody>
</table>

1 n = 67 girls and 59 boys. Percentages in brackets.
2–4 Significantly different from baseline (paired t tests with Bonferroni correction): 2P < 0.05, 3P < 0.01, 4P < 0.001.

increased the prevalence of anemia from 0% to 43% at 15 mo. The iron status indicators worsened: there was a significant decrease in mean SF (P < 0.01) and an increase in mean TIR (P < 0.01). Mean body iron stores in the children fell by −5.2 mg/kg (P < 0.001). At 15 mo, three-quarters of the cohort had deficits in tissue iron, and one-third had IDA. In the regression models, changes in height, weight, and BSA were not significantly correlated with change in body iron (in mg/kg) from 0 to 15 mo.

The components of iron balance in the cohort over the 15 mo study are shown in Table 4. The mean overall decrease in total body iron was −142 mg, because of a modest decrease in hemoglobin iron, a sharp decrease in iron stores, and a slight increase in nonstorage tissue iron. Total mean dietary iron absorption over 15 mo was 99 mg, or 0.22 mg per day. Because mean dietary intake of iron was 10.8 mg/d (Table 1), only 2% of the iron in the local diet was absorbed during the 15-mo period. Cook et al (27) reported IDA developed in adults at a deficit in iron stores of −4 mg/kg. Our data indicate children develop mild anemia even with smaller deficits of tissue iron stores (Figure 1). Among the 54 children with anemia in the cohort at 15 mo (3 ± SD hemoglobin = 108 ± 4 g/L), although all had deficits in iron stores, the mean iron deficit was only −1.1 mg/kg.

DISCUSSION

This is the first prospective, longitudinal cohort study in a developing country to assess the effect of habitual diets low in bioavailable iron on iron status. Strengths of the study included the following: 1) a moderately large sample size limited to a discrete population group vulnerable to IDA, 2) a cohort free of parasitic infections that cause blood iron losses and confound measurement of SF, 3) detailed characterization of the local diet with supervised weighed food records in 2 seasons in randomly selected families and direct analysis of iron and phytic acid content of principal foods, and 4) reliable quantitative laboratory measurement of iron status, including SF, TIR, and body iron stores.

Our findings demonstrate that low iron bioavailability from a legume and cereal–based diet can be a cause of iron deficiency in children in rural Africa. The children in the cohort had sufficient dietary energy and protein, and their diet contained 5.4 mg iron/1000 kcal. This is near the usual iron content of typical Western diets, ≈6 mg/1000 kcal (2), and is consistent with data from food balance sheets for Africa from the Food and Agriculture Organization (30) reporting per capita iron intake of 14–21 mg/d. Because the absorbed iron requirement for this age group is 0.7–0.8 mg/d (2), the children’s iron intake was many times higher than their iron requirement. They would have needed to absorb ≈8% of their dietary iron to meet their requirements, but they only absorbed ≈2%, or 0.23 mg/d. Iron balance was negative over the 15 mo, and most of the children exhausted their iron stores and developed iron deficiency.

![Figure 1](image-url)
There are several potential limitations to the findings in this study. First, the accuracy and precision of food records to define nutrient intake could be limited by sampling bias, variation, and measurement error. The families participating in the food survey could have modified their usual food habits, so that the intake data might not have accurately represented the habitual diet. However, well-trained, local surveyors emphasized the need to maintain usual diets and did the weighing and recording in all households. The households were randomly selected and none declined participation. Three-day weighed records can be an accurate method for assessing dietary intake of specific nutrients (31), and both the within- and between-subject variations in daily iron intake were lower than those generally reported from Western countries, as a result of the day-to-day uniformity of the local diet (31). Second, our iron balance calculations were based on several assumptions. We calculated basal losses with use of a formula derived in adults and adjusted for BSA (2). This approach might underestimate basal losses in younger children, because it gives lower values than gastrointestinal losses of iron reported in infant studies (32). There could be additional losses of iron in the cohort from occult bleeding or infection or both that went undetected. If iron losses were underestimated, our calculations would have underestimated the amount of dietary iron absorbed. Although we assumed that nonstorage tissue iron would increase, IDA can reduce the activity of iron-dependent enzymes (33), but this component contributes only minimally to iron needs. The cohort included only children with a negative CRP to minimize the confounding effect of inflammation on SF, but the timing of the rise and fall of CRP during the acute-phase response differs from that of SF. It is possible that SF was elevated by the acute-phase response in some children despite a negative CRP. Finally, the algorithm of Cook et al (27) for calculating body iron stores is derived from an adult phlebotomy study and is not validated in children. However, in a recent iron fortification trial in children it performed well (34).

Dietary iron in northern Morocco is poorly absorbed because concentrations of dietary MFP and ascorbic acid are low, and the phytic acid content is high. Legumes and cereals are the dietary staples, providing two-thirds of dietary energy, and both are rich in phytic acid. Mean daily intake of phytic acid was 1.7 g, compared with the usual phytic acid content of 0.2–0.8 g/d in Western diets (35). Phytic acid is a strong inhibitor of iron absorption. Hallberg et al (36) found that adding 20 mg phytic acid/100 g to bread rolls decreased iron absorption by 40%. In northern Morocco, bread made from the local wheat flour contains 350 mg phytic acid/100 g dry weight. The 2% absorption of dietary iron in this study is consistent with results from isotope-labeled single meal studies in which iron absorption (both of native iron and fortification iron) was as low as 1–3% from meals based on whole-grain cereals and legumes, even in iron-deficient subjects (37–39). It is lower than the usual estimate of ~5% iron bioavailability from plant-based diets in developing countries.

Ascorbic acid is an enhancer of iron absorption in the presence of phytic acid (37). The magnitude of the effect depends on the amount of ascorbic acid and the food matrix. An ascorbic acid-to-iron molar ratio of ≥2:1 will usefully increase the absorption of soluble iron from low phytate foods, but a ratio of ≥4:1 is needed to increase iron absorption from diets high in phytic acid (40). In the present study, the molar ratio of ascorbic acid to iron was 1:1. Actual intakes of ascorbic acid were likely lower than estimated; ascorbic acid is susceptible to losses during food storage and food preparation, and most Moroccan dishes containing vegetables (the source of >80% of dietary ascorbic acid) are simmered for long periods. Muscle tissue is also a strong enhancer of nonheme-iron absorption (3). Although mean daily consumption of MFP in the children was 56 g, more than two-thirds was in the form of sardines, which are estimated to have a heme iron content of only 20%. Compared with industrialized countries, where ~10–15% of iron intake is heme iron (41), in the present study <3% of dietary iron was heme iron. Nevertheless, because of its high absorption, we estimated heme iron contributed up to 30% of the total amount of iron absorbed.

We used 2 algorithms to estimate dietary iron absorption in the present study (20, 21). Of the 2 models, based on our calculation of absorbed iron, the model of Reddy et al (21) better predicted iron absorption from the local diet. When adjusted for low iron stores, it predicted an iron absorption of 0.21 mg/d, in close agreement with the calculated mean absorption of 0.22 mg/d in the cohort. Reddy et al proposed their model could predict nonheme-iron absorption from meals typical of Western diets. Although it was criticized as unsuitable for diets in developing countries (42), it performed well when applied to the northern Moroccan diet, even though the local phytic acid content was much higher than in the Western meals from which the algorithm was derived (21). A variable not considered by these absorption models is vitamin A status. Vitamin A deficiency can impaire iron metabolism and bioavailability (43). The children in the cohort were marginally vitamin A deficient, and 12% of children had serum retinol concentrations <0.7 μmol/L. Although the severity of vitamin A deficiency was only mild, it is possible that poor vitamin A status also contributed to low iron bioavailability in the cohort.

Our data highlight the challenges faced by iron fortification programs in developing countries where the diet is low in iron bioavailability. To meet a local Moroccan child’s iron requirements, a food fortification program would need to supply ~0.5 mg additional absorbed iron daily. Without a change in dietary quality, and assuming fortification iron enters the common pool and is absorbed at a level of 2%, 23 mg additional iron per day would be needed. The current flour fortification program in Morocco calls for 45 mg iron as electrolytic iron/kg flour. Assuming a relative bioavailability of 50% for electrolytic iron compared with iron in the common pool (44), a child in northern Morocco would need to consume nearly 1 kg flour/d to meet his or her needs. These data emphasize that the mere addition of iron to staple foods in developing countries, without providing an enhancer of iron absorption, is unlikely to have significant effect on iron status. The findings underscore the need for new approaches to improve iron bioavailability in food fortification.

We thank the participating children and teachers as well as the staff at the Brikcha Health Center. Special thanks goes to R Rahmouni (Brikcha, Morocco), M El-Yazami and M Saissi (Chefchaouen, Morocco), and C Zeder, R Wegmüller, A Khatir, and S Kollart (Swiss Federal Institute of Technology, Zürich).

Each of the authors contributed to the study design. MBZ and NC performed the fieldwork and the data collection. MBZ and RFH supervised the laboratory analysis and completed the data analysis. MBZ conducted the statistical analysis. The first draft of the manuscript was written by MBZ. All authors edited the manuscript. None of the authors had a financial or personal conflict of interest in regard to this study.
REFERENCES


Ingestion of a tea rich in catechins leads to a reduction in body fat and malondialdehyde-modified LDL in men\textsuperscript{1–3}

Tomonori Nagao, Yumiko Komine, Satoko Soga, Shinichi Meguro, Tadashi Hase, Yukitaka Tanaka, and Ichiro Tokimitsu

**ABSTRACT**

**Background:** Catechins, the major component of green tea extract, have various physiologic effects. There are few studies, however, on the effects of catechins on body fat reduction in humans. It has been reported that the body mass index (BMI) correlates with the amount of malondialdehyde and thiobarbituric acid–reactive substances in the blood.

**Objective:** We investigated the effect of catechins on body fat reduction and the relation between oxidized LDL and body fat variables.

**Design:** After a 2-wk diet run-in period, healthy Japanese men were divided into 2 groups with similar BMI and waist circumference distributions. A 12-wk double-blind study was performed in which the subjects ingested 1 bottle oolong tea/d containing 690 mg catechins (green tea extract group; n = 17) or 1 bottle oolong tea/d containing 22 mg catechins (control group; n = 18).

**Results:** Body weight, BMI, waist circumference, body fat mass, and subcutaneous fat area were significantly lower in the green tea extract group than in the control group. Changes in the concentrations of malondialdehyde-modified LDL were positively associated with changes in body fat mass and total fat area in the green tea extract group.

**Conclusion:** Daily consumption of tea containing 690 mg catechins for 12 wk reduced body fat, which suggests that the ingestion of catechins might be useful in the prevention and improvement of lifestyle-related diseases, mainly obesity. *Am J Clin Nutr* 2005; 81:122–9.

KEY WORDS Green tea extract, catechins, humans, body fat, malondialdehyde-modified LDL, double-blind controlled study

**INTRODUCTION**

High body fat increases the risk of diabetes, hyperlipidemia, and hypertension, which leads to arteriosclerotic disease. There is an increased risk of death associated with these diseases as well as with increased body fat (1–3). Body fat increases with an increase in dietary lipid intake (4, 5). Therefore, recommendations of lifestyle changes, and of changes in dietary content in particular, are often made for primary prevention and improvement of these diseases.

Polyphenols have recently attracted attention because of their physiologic activity. Green tea, long consumed in Asian countries (mainly Japan and China), contains low-molecular-weight polyphenols consisting mainly of flavanol (flavan-3-ol) monomers, which are referred to as catechins. There are several isomers of this compound: catechin, catechin gallate (Cg), gallo catechin, gallo catechin gallate (GCg), epicatechin, epicatechin gallate (ECg), epigallocatechin, and epigallocatechin gallate (EGCg). Normally, 10–20% of the catechins in green tea leaves are epigallocatechin and EGCg (6). A portion of ingested EGCg is absorbed and widely distributed throughout the body (7). The ingestion of tea extract or catechins induces antioxidant (8), antiviral (9), antiplaque-forming (10), and anticancer (11) activities, as well as decreases in blood pressure (12) and blood sugar (13). Lipid metabolism studies in animals, tissues, and cells have found that tea extract and catechins reduce triacylglycerol and total cholesterol concentrations (14, 15), inhibit hepatic and body fat accumulation (16, 17), and stimulate thermogenesis (18). In humans, there have been few studies on the effects of catechins on body fat, but the effects on energy expenditure (EE) and oxidative consumption have been examined (19, 20). One study of the effects of catechins on body weight found a tendency toward decreased body weight and waist circumference, but no comparative controls were included in the study (21).

Studies reported that body mass index (BMI; in kg/m\textsuperscript{2}) correlates with the amount of thiobarbituric acid–reactive substances (TBARS) and malondialdehyde in the blood (22, 23). These studies suggested that obesity might be related to an increase in lipid oxidizability. To examine the hypothesis that continuous ingestion of catechins reduces body fat in humans, we performed a double-blind controlled study in healthy men (n = 38) who were normal-weight to overweight and in whom the intake of catechins and caffeine from other foods was minimized. The effects of catechins on the blood variables and oxidized LDL and the association between body fat variables and oxidized LDL were investigated.

**SUBJECTS, MATERIALS, AND METHODS**

**Subjects**

The subjects were 38 male employees of Kao Corporation (Tokyo), aged 24–46 y, whose body weight was normal to overweight, according to the criteria of the National Institutes of Health for BMI (24).

\textsuperscript{1} From Health Care Products Research Laboratories No.1, Kao Corporation, Tokyo (TN, YK, SS, SM, TH, YT, and IT).

\textsuperscript{2} Supported by Kao Corporation.

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The study was performed under the supervision of an occupational health physician, in accordance with the regulations of the Kao Corporation Ethics Committee for Internal Clinical Studies and in conformity with the Helsinki Declaration. The conditions and procedures of the investigation were reviewed with all subjects before they gave written informed consent.

Materials

Green tea extract (GTE) was prepared by using 2 extraction methods. To prepare one version (GTE-A), green tea leaves underwent extraction with hot water, and the extract was reduced to a powder by using the spray-dry method. The powder was dissolved in methanol-water, discolored with the use of an octadecyl silica column, and freeze-dried (10). To prepare another version (GTE-B), which is decaffeinated, the GTE obtained with hot water was reduced to a powder by using the spray-drying method, and then the extract was dissolved in hot water and mixed with an equal volume of chloroform. The aqueous phase was recovered with 3 volumes of ethanol, and the extract was freeze-dried after removal of the solvent (10). The total catechin content was 33.0 g/100 g in the GTE-A powder and 85.6 g/100 g in the GTE-B powder. For preparation of the test beverage, 9 g oolong tea leaves underwent extraction with 100 mL distilled water at 80 °C for 5 min, and the extract was used as the base beverage. Two types of beverage were prepared: one beverage containing a high amount of catechins and a control beverage. The beverage with a high catechin content was prepared by adding both GTEs to the base beverage and adjusting the total catechin content to ≈700 mg/340 mL base beverage. For the control beverage, both GTEs were added to the base beverage, and the total catechin content was adjusted to ≈20 mg/340 mL base beverage; this is the same ratio of catechin components to the caffeine content as in the beverage with a high catechin content. To avoid oxidation and to maintain quality throughout the study period, 50 mg ascorbic acid/100 mL beverage was added to the beverages, and the beverages were sterilized by high-temperature heat sterilization at 138 °C for 30 min. Aliquots of 340 mL were distributed in 340-mL polyethylene terephthalate bottles.

The amounts of catechin, Cg, gallate catechin, GCg, epicatechin, ECg, epigallocatechin, and EGCG in the test beverage were measured by using HPLC carried out on an L-column ODS (4.6 mm diameter × 250 mm length; Chemicals Evaluation and Research Institute, Tokyo). The HPLC conditions were as follows: column temperature, 35 °C; sample size, 10 μL (0.1 mol acetic acid solution/L); mobile phase, 0.1 mol acetic acid/L (solution A); 0.1 mol acetic acid-acetonitrile/L (solution B); gradient conditions, 0–5 min ratio of solution A to solution B = 97:3, 5–37 min A:B = 80:20, 37–43 min A:B = 80:20, 43–43.5 min A:B = 100:0; and 43.5–48.5 min A:B = 100:0; flow rate, 1.0 mL/min; and measurement wavelength, 280 nm. Catechin, Cg, gallatecatechin, GCg, epicatechin, ECg, epigallocatechin, and EGCG in proportions of 40, 100, and 200 μg/mL with a purity of ≥98% (Kurita Water Industries Ltd, Tokyo) in 0.1 mol acetic acid-methanol/L were used as standards. The calibration curves were prepared from the peak area of 10 μL of each standard solution.

The water content of the test beverage was ascertained by measuring the weight before and after drying at atmospheric pressure and at 105 °C by using a forced-circulation warm-air dryer. The protein content of the test beverage was calculated by using nitrogen × 6.25, and the amount of nitrogen was measured by using the Kjeldahl method. For protein quantification, the amount of nitrogen in caffeine (amount of caffeine × 56/194, with 56 and 194 being the MW of 4 nitrogens and caffeine, respectively) was subtracted from the total amount of nitrogen.

For lipid quantification, the test beverage was deproteinized by using a 7% copper sulfate solution, and the pH was adjusted to 6–7; the sample then underwent extraction with diethylether for 16 h with the use of a Soxhlet extractor. The lipid content of the test beverage was ascertained by measuring the weight before and after removal of the solvent. The ash content was ascertained by measuring the weight of the sample with the use of the direct ash method by heating at 550 °C.

Dietary fiber was quantified by using the enzyme-weight method. Dried powder of the test beverage (10 g) was combined with 40 mL phosphate buffer (0.08 mol/L; pH 6.0) and 0.1 mL of a thermostable α-amylase (Termamyl 120 L; Novo-Nordisk A/S, Bagsværd, Denmark), which was then incubated in a boiling water bath for 30 min. After cooling, the solution was adjusted to pH 7.5 ± 0.1 with the use of sodium hydroxide and combined with 0.1 mL of 50 mg protease/mL (product no. P-5380; Sigma-Aldrich Co, St Louis) in phosphate buffer (pH 6.0), and the mixture was incubated at 60 °C for 30 min. After cooling, the solution was adjusted to pH 4.3 ± 0.3 with the use of hydrochloric acid and combined with 0.1 mL amyllogloosidase solution (product no. A-9913, Sigma-Aldrich Co), and the mixture was incubated at 60 °C for 30 min. Next, the solution was combined with 4 volumes of 95% ethanol at 60 °C, kept at room temperature for 1 h, and filtered. The residue was washed 3 times with 78% ethanol, ≥2 times with 95% ethanol, and ≥2 times with acetone, and then it was dried at 105 °C overnight, and the weight was measured. This procedure was repeated twice—first for measurement of the ash content after incineration treatment and again for protein quantification by using the Kjeldahl method. The dietary fiber content was calculated by subtracting the protein content from the ash content.

Sodium was measured by atomic absorption spectrometry at a wavelength of 589.6 nm with the use of an acetylene-air flame. To quantify the sodium content, the dried powder of the test beverage was combined with 1% HCl and allowed to stand overnight.

For ascorbic acid measurement, a 10% solution was filtered, and 1 mL of the filtrate was combined with 1 mL of 5% metaphosphoric acid. This solution was oxidized by combining the sample with a few drops of 0.2% dichlorphenolindophenol and 2 mL of 2% thiourea–5% metaphosphoric acid, which was followed by the addition of 0.5 mL of 2% 2,4-dinitrophenylhydrazine in 4.5 mol sulfuric acid/L. This solution was then heated at 40 °C for 16 h to produce osazone and combined with 3 mL ethyl acetate; the sample was shaken for 1 h and quantified by using HPLC. The HPLC conditions were as follows: column, Silica-1100-N (4.6 mm diameter × 100 mm length; Senshu Scientific Co, Ltd, Tokyo), column temperature, 35 °C; mobile phase, ethyl acetate:hexane:acetic acid:water = 60:40:5:0.05; flow rate, 1.5 mL/min; and measurement wavelength, 495 nm.

Caffeine was quantified by using HPLC after the dried powder was dissolved in methanol and adjusted to the specified volume. The HPLC conditions were as follows: column, Mightyssil RP-18 Aqua (5 μm particle size; 4.5 mm × 150 mm diameter; Kanto Kagaku, Tokyo); column temperature, 50 °C; mobile phase, the ratio of 0.03 mol acetate buffer/L (pH 4) to acetonitrile = 850:30;
flow rate, 2.0 mL/min; and measurement wavelength, 270 nm. Tannin was measured as tannic acid by using the Folin-Denis method.

Carbohydrates were calculated as the residue of these substances. Energy was calculated by using the energy conversion coefficients (ie, protein, 16.7 kJ/g; lipid, 37.7 kJ/g; and carbohydrate, 16.7 kJ/g).

**Protocol**

Before the study, all subjects were taught to calculate their energy and lipid intakes by using 2 guidebooks, the *Standard Tables of Food Consumption in Japan, 4th edition* (25), and the *New Calorie Guide Book for Daily Diet* (26). The energy intake (EI) required for living activity strength 1 (a Japanese term, equivalent to a physical activity level of 1.3), based on the subject’s advance activity report, was calculated by using the equation below from the standard table (25):

\[ A \text{ (kJ/d)} = 1.35B \times 10.9 \quad (I) \]

where \( A \) is measured in kJ/d, \( B = a \times \text{weight (kg)}^{0.444} \times \text{height (cm)}^{0.663} \times 88.83/10000 \times 24 \times 4.184, \) and \( a \text{ (kcal/m}^2 \cdot \text{h}^{-1}) \) is the standard value of basal metabolic rate at living activity strength 1 (37.5 for age 20–29 y; 36.5 for age 30–39 y; and 35.6 for age 40–49 y).

During the study period, the subjects were instructed to ingest 90% of the individual EI calculated above and to ingest 60 g lipids/d, which is the average intake in Japan, as calculated from the 1998 National Nutrient State report (27). They were instructed to stay within ± 10% of those amounts.

On weekdays (Monday–Friday), the subjects ate the same set menu for breakfast between 0730 and 0830 and the same set menu for supper between 1700 and 1800 at the company cafeteria. For lunch, the subjects could select one of several dishes on the cafeteria menu that were controlled for EI and lipid intake within the standard ranges given above. Lunch was eaten between 1130 and 1330. On weekends, holidays, and other days when they were unable to eat at the company cafeteria, the subjects controlled their own daily EI and lipid intake within the standard ranges by using the guidebooks.

The consumption of foods and beverages containing large amounts of catechins, polyphenols, or caffeine, such as green tea, oolong tea, wine, and coffee, was prohibited under the protocol. The subjects were allowed to drink ≤27.5 mL alcohol/d, which equals ≈500 mL beer/d. The subjects were advised to maintain their current level of exercise.

Anthropometry, computed tomography (CT), and inquiries by the occupational health physician were performed every 4 wk. Body weight, waist circumference, and hip circumference measurements were performed by using the caliper method. Body fat mass and lean body mass were calculated from the ratio of body weight to body fat. Waist circumference at the umbilical level was measured while the subjects were standing, according to the criteria of the Japan Society for the Study of Obesity. For skinfold-thickness measurements, the subcutaneous fat thickness was measured at the lower end of the scapula on the back and in the intermediate region on the extensor side of the arm, and the sum of the 2 values was calculated.

**Anthropometric measurements**

Body weight, waist circumference, and hip circumference were measured. The body fat ratio was measured by using the bioimpedance analysis method (Tanita Body Fat Analyzer, model no. TBF-401; Tanita Co, Tokyo), and skinfold-thickness measurements were performed by using the caliper method. Body fat mass and lean body mass were calculated from the ratio of body weight to body fat. Waist circumference at the umbilical level was measured while the subjects were standing, according to the criteria of the Japan Society for the Study of Obesity. For skinfold-thickness measurements, the subcutaneous fat thickness was measured at the lower end of the scapula on the back and in the intermediate region on the extensor side of the arm, and the sum of the 2 values was calculated.

**Measurement of fat by computed tomography**

Within 3 d of the anthropometric measurements, the subjects underwent CT imaging (TCT-300; Toshiba Medical Co, Tokyo) of the abdominal transverse section at the L4–L5 level at the Yabuki Clinic (Tochigi, Japan). CT imaging was performed under the conditions for visceral fat measurement by using FAT SCAN software (version 2; N2 System Co, Osaka, Japan), which was developed on the basis of the method reported by Tokunaga et al (28). The X-ray conditions were tube voltage of 120 kVp and mAs of 360; the film was processed at a window level of 0 and a window width of 1000. By using the abovementioned software, the visceral fat area (VFA) and subcutaneous fat area (SFA) were obtained from the abdominal CT image, and these areas were summed to obtain the total fat area (TFA).

**Blood sampling and clinical analysis**

Alcohol consumption was prohibited starting 3 d before blood sampling, and eating or drinking anything other than water was prohibited after 2100 the day before sampling. Fasting blood was collected from a vein on the flexor side of the arm between 0900 and 1000. The blood samples were analyzed by SRL Inc (Tokyo).

Concentrations of the following variables were measured in week 0 and week 12: triacylglycerol (Pureauto STG-N; Daiichi Pure Chemicals Co, Ltd, Tokyo), total cholesterol (L type Wako CHO - H; Wako Pure Chemicals Co, Ltd, Osaka, Japan), HDL cholesterol (Cholestest HDL; Daiichi Pure Chemicals Co, Ltd), LDL cholesterol (Cholestest LDL; Daiichi Pure Chemicals Co, Ltd), remnant-like lipoprotein cholesterol (RLP-cholesterol JIMRO II; Japan Immunoresearch Laboratories Co, Ltd, Gunma, Japan), free fatty acid [(FFA) NEFA-SS EIKEN; Eiken Chemical Co, Ltd, Tokyo], total ketone body (Total ketone body KAI-NOS; KAINOS Laboratories Inc, Tokyo), blood sugar (Quick auto neo GLU-HK; Shino-Test Co, Tokyo), insulin (EIKEN Insulin; Eiken Chemical Co, Ltd), leptin (Human Leptin RIA Kit; Linco Research Inc, St Charles, MO), and total plasminogen activator inhibitor-1 ([PAI-1] LPIA - iPAI test; Dia-Iatron Co, Ltd, Tokyo). We measured vitamin A by using a modification of the method reported by Henry et al (29): the serum sample was
combined with methanol, mixed, and centrifuged. The supernatant was separated by ODS reversed-phase HPLC, and vitamin A was detected at excitation wavelengths of 325 nm and emission wavelengths of 480 nm. We measured vitamin E by using the method of Abe et al (30). We also measured malondialdehyde-modified LDL [(MDA-LDL) by using an enzyme-linked immunosorbent assay using anti–MDA-LDL antibody (ML25; 31)], aspartate transaminase (Transaminase-HR II; Wako Pure Chemical Co, Ltd), glutamic-pyruvic transaminase (Transaminase-HR II; Wako Pure Chemical Co, Ltd), blood urea nitrogen (Pureauto S UN; Daiichi Pure Chemicals Co, Ltd), calcium (Orthocresol-REDUCTION OF BODY FAT AND MDA-LDL BY CATECHINS 125

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Composition of test beverages(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control beverage(^2)</td>
</tr>
<tr>
<td>Total catechin (mg/100 mL)</td>
<td>6.4 (100.0)</td>
</tr>
<tr>
<td>Catechin (mg/100 mL)</td>
<td>0.5 (7.8)</td>
</tr>
<tr>
<td>Epicatechin (mg/100 mL)</td>
<td>0.3 (4.7)</td>
</tr>
<tr>
<td>Catechin gallate (mg/100 mL)</td>
<td>0.2 (3.1)</td>
</tr>
<tr>
<td>Epicatechin gallate (mg/100 mL)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>Galloatechin (mg/100 mL)</td>
<td>1.7 (26.6)</td>
</tr>
<tr>
<td>Epigallocatechin (mg/100 mL)</td>
<td>1.1 (17.2)</td>
</tr>
<tr>
<td>Galloatechin gallate (mg/100 mL)</td>
<td>1.7 (26.6)</td>
</tr>
<tr>
<td>Epigallocatechin gallate (mg/100 mL)</td>
<td>0.9 (14.0)</td>
</tr>
<tr>
<td>Water (g/100 mL)</td>
<td>99.5</td>
</tr>
<tr>
<td>Protein (g/100 mL)</td>
<td>0.1</td>
</tr>
<tr>
<td>Fat (g/100 mL)</td>
<td>0.0</td>
</tr>
<tr>
<td>Carbohydrate (g/100 mL)</td>
<td>0.3</td>
</tr>
<tr>
<td>Dietary fiber (g/100 mL)</td>
<td>ND</td>
</tr>
<tr>
<td>Ash (g/100 mL)</td>
<td>ND</td>
</tr>
<tr>
<td>Sodium (mg/100 mL)</td>
<td>10</td>
</tr>
<tr>
<td>Ascorbic acid (mg/100 mL)</td>
<td>50</td>
</tr>
<tr>
<td>Tannin (mg/100 mL)</td>
<td>10</td>
</tr>
<tr>
<td>Caffeine (mg/100 mL)</td>
<td>23</td>
</tr>
<tr>
<td>Calories (kJ/100 mL)</td>
<td>6.7</td>
</tr>
</tbody>
</table>

\(^1\) ND, not detectable.
\(^2\) Percentage of total catechin in parentheses.

Statistical analysis

All evaluation variables were presented as means ± SEMs. For statistical analysis, STATVIEW for WINDOWS software (version 4.58; Abacus Concepts, Berkeley, CA) was used. Baseline measurements were assessed by using the unpaired t test. The effect of the GTE was assessed by using two-factor repeated-measures analysis of variance (ANOVA), and variables were measured each week. To compare the magnitude of the decreases in the anthropometric values and in body composition at the endpoint, the differences between the values at the initial measurement and those at week 12 were analyzed by using an unpaired t test. Linear regression analysis was used to evaluate the association between MDA-LDL and body fat variables at week 12. A P value < 0.05 was considered to be significant.

RESULTS

Analysis of the test beverages

The contents and analyzed ingredient values of catechin, Cg, gallocatechin, GCg, epicatechin, ECg, epigallocatechin, and EGCg in the test beverages are shown in Table 1. The total catechin content was 689.9 mg/340 mL beverage in the high-catechin beverage and 21.8 mg/340 mL in the control beverage.

Dietary condition of the subjects

Three subjects were excluded from the analysis because of a marked change in their living environment during the study (control group, n = 18; GTE group, n = 17). The daily EI was 8.7 ± 0.1 MJ at week 0 and 8.8 ± 0.1 MJ at week 12 in the control group and 8.6 ± 0.1 MJ at week 0 and 8.6 ± 0.1 MJ at week 12 in the GTE group. Those daily energy values were very close to the Japanese standard, which is 9.0–9.4 MJ in men 20–40 y old at living activity strength 1 (35), and 90% of the values were 8.3–8.5 MJ. The mean (± SE) daily lipid intake was 59.2 ± 0.8 g at week 0 and 61.4 ± 0.9 g at week 12 in the control group and 61.5 ± 1.4 g at week 0 and 59.6 ± 0.8 g at week 12 in the GTE group. There were no significant differences between the 2 groups. The mean values of each variable were calculated every 4 wk and analyzed by using a two-factor repeated-measures ANOVA, and there was no significant difference between groups.

Effects on anthropometric values and body composition

Because one subject in the control group did not consent to CT imaging at weeks 4 and 8, analyses of the control group for weeks 4 and 8 were performed in 17 subjects. There was no significant difference in the initial value of any variable between the 2 groups (Table 2). There was a significant time-by-group interaction for body weight, BMI, waist circumference, body fat mass, and SFA (Table 2). The decrease in waist circumference between the initial measurement and that at week 12 in the GTE group was significantly greater than that in the control group (−3.4 ± 0.5 cm and −1.6 ± 0.4 cm, respectively; Table 2). The decrease in skinfold thickness between the initial measurement and that at week 12 in the GTE group was significantly greater than that in the control group (−3.3 ± 0.7 mm and −1.3 ± 0.7 mm, respectively; Table 2). The decrease in TFA between the initial measurement and that at week 12 in the GTE group was significantly greater than that in the control group (−26.7 ± 6.0 cm² and −6.7 ± 5.8 cm², respectively; Table 2). The decrease in SFA between the initial measurement and that at week 12 in the GTE group was significantly greater than that in the control group (−16.7 ± 3.0 cm² and −4.4 ± 4.1 cm², respectively; Table 2).

Effects on blood variables and side effects

There was no significant difference between the groups in the initial value of any variable (Table 3). There was a significant
time-by-group interaction for vitamin E and MDA-LDL concentrations (Table 3). Among the variables for which standard values for Japanese have been established (36), the mean values did not deviate from the standard values throughout the study period.

Table 2: Changes in anthropometric variables and body composition after consumption of either control or high-catechin beverages for 12 wk

<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>4 wk</th>
<th>8 wk</th>
<th>12 wk</th>
<th>Change at 12 wk</th>
</tr>
</thead>
</table>
| Weight (kg)
| Control group           | 73.8 ± 1.3 | 72.9 ± 1.3 | 72.7 ± 1.4 | 72.5 ± 1.4 | −1.3 ± 0.3     |
| GTE group                | 73.9 ± 1.8 | 72.6 ± 1.7 | 72.2 ± 1.7 | 71.5 ± 1.7 | −2.4 ± 0.5     |
| BMI (kg/m²)
| Control group           | 25.0 ± 0.4 | 24.7 ± 0.4 | 24.6 ± 0.4 | 24.6 ± 0.4 | −0.4 ± 0.1     |
| GTE group                | 24.9 ± 0.4 | 24.4 ± 0.4 | 24.3 ± 0.4 | 24.1 ± 0.4 | −0.8 ± 0.2     |
| Waist (cm)
| Control group           | 87.8 ± 1.1 | 86.7 ± 1.1 | 86.6 ± 1.1 | 86.2 ± 1.2 | −1.6 ± 0.4     |
| GTE group                | 87.9 ± 1.4 | 86.6 ± 1.4 | 85.5 ± 1.3 | 84.5 ± 1.3 | −3.4 ± 0.5     |
| Hip (cm)
| Control group           | 97.0 ± 0.8 | 95.9 ± 0.7 | 95.8 ± 0.8 | 95.8 ± 0.8 | −1.1 ± 0.3     |
| GTE group                | 97.4 ± 0.9 | 96.0 ± 1.1 | 96.1 ± 1.1 | 96.1 ± 1.1 | −1.3 ± 0.3     |
| Body fat mass (kg)
| Control group           | 19.5 ± 1.0 | 18.8 ± 0.9 | 18.8 ± 1.0 | 18.8 ± 1.1 | −0.7 ± 0.3     |
| GTE group                | 19.7 ± 0.8 | 19.2 ± 0.9 | 18.0 ± 0.9 | 18.3 ± 0.9 | −1.4 ± 0.3     |
| Lean body mass (kg)
| Control group           | 54.3 ± 0.7 | 54.1 ± 0.7 | 53.9 ± 0.7 | 53.7 ± 0.7 | −0.6 ± 0.3     |
| GTE group                | 54.2 ± 1.1 | 53.4 ± 1.0 | 54.1 ± 1.1 | 53.2 ± 1.0 | −1.0 ± 0.4     |
| Skinfold thickness (mm)
| Control group           | 27.0 ± 1.5 | 25.3 ± 1.3 | 26.2 ± 1.5 | 25.7 ± 1.4 | −1.3 ± 0.7     |
| GTE group                | 27.9 ± 1.8 | 26.3 ± 1.6 | 25.9 ± 1.8 | 24.6 ± 1.5 | −3.3 ± 0.7     |
| TFA (cm²)
| Control group           | 261.0 ± 12.7 | 254.2 ± 13.1 | 246.4 ± 12.7 | 254.3 ± 13.6 | −6.7 ± 5.8     |
| GTE group                | 258.4 ± 11.0 | 246.3 ± 11.2 | 232.1 ± 9.9 | 231.7 ± 11.1 | −26.7 ± 6.0    |
| VFA (cm²)
| Control group           | 89.3 ± 5.8 | 88.9 ± 6.5 | 84.5 ± 5.0 | 87.0 ± 5.2 | −2.4 ± 2.7     |
| GTE group                | 83.1 ± 5.7 | 79.2 ± 5.4 | 73.2 ± 5.3 | 73.0 ± 5.3 | −10.1 ± 4.0    |
| SFA (cm²)
| Control group           | 171.7 ± 10.7 | 165.3 ± 10.7 | 161.9 ± 11.0 | 167.3 ± 11.0 | −4.4 ± 4.1     |
| GTE group                | 175.3 ± 8.2 | 167.1 ± 8.7 | 158.8 ± 8.3 | 158.7 ± 7.9 | −16.7 ± 3.0    |

1 All values are x ± SEM. GTE, green tea extract; TFA, total fat area; VFA, visceral fat area; SFA, subcutaneous fat area. Control group, n = 18; GTE group, n = 17. The initial values did not differ significantly between groups. Data from weeks 0, 4, 8, and 12 were compared by using two-factor repeated-measures ANOVA with time and group.
2 Significant effect of time from week 0 to week 12, P < 0.01.
3 Significant time-by-group interaction, P < 0.05.
4, 5 Significant difference between groups for change at 12 wk (unpaired t test); 4P < 0.01, 5P < 0.05.
6 Control group, n = 18 at initial measurement and 12 wk; n = 17 at 4 and 8 wk.

Effects on blood MDA-LDL and the association between MDA-LDL and body fat variables

Because the initial MDA-LDL values tended to differ between groups, the variable was compared on the basis of the change by week 12, and we took 100% as the initial value. The decrease in MDA-LDL between the initial measurement and that at week 12 in the GTE group was significantly greater than that in the control group (67.5 ± 6.1% and 89.5 ± 6.0%, respectively; P < 0.05). To investigate the association between MDA-LDL and body fat variables, linear regression analysis was performed within each group. The percentage value of MDA-LDL in week 12 was used as an independent variable, and the percentage values of each anthropometric variable and abdominal fat area in week 12 were used as dependent variables. In the GTE group, the percentage value of MDA-LDL at week 12 was significantly associated with the percentage values of body fat mass (R² = 0.646, P = 0.0001) and TFA (R² = 0.273, P = 0.0313) (Figure 1). In the control group, however, those associations were not observed (P > 0.05).

DISCUSSION

In the current study, the samples were prepared in beverage form so that the subjects could easily consume them every day for 12 wk. The base beverage was oolong tea, which is the most widely sold tea in Japan (37). The average consumption of green tea and of catechins in Japan is calculated to be ≈2 g tea leaves/d and 200–400 mg catechin/d (6). Before conducting this study, for clarification of the effect of catechins, the caffeine content in the test beverages was adjusted to a minimal amount, and the consumption of beverages and foods containing large amounts of catechins or caffeine was prohibited so as to minimize the effect of other food-derived catechins and caffeine. Dietary control, aimed at weight loss for both groups, was included as a benefit for subjects in the control group: the standard daily EI was set to 90% of the value calculated from the body surface area and daily living.
Table 3

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>GTE group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial (n = 18)</td>
<td>12 wk</td>
</tr>
<tr>
<td><strong>Triacylglycerol (mmol/L)</strong></td>
<td>1.38 ± 0.11</td>
<td>1.41 ± 0.12</td>
</tr>
<tr>
<td><strong>Cholesterol (mmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.77 ± 0.16</td>
<td>5.07 ± 0.18</td>
</tr>
<tr>
<td>HDL</td>
<td>1.23 ± 0.07</td>
<td>1.30 ± 0.08</td>
</tr>
<tr>
<td>LDL</td>
<td>3.27 ± 0.13</td>
<td>3.10 ± 0.14</td>
</tr>
<tr>
<td>RLP</td>
<td>0.11 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>Free fatty acid (mmol/L)</td>
<td>0.44 ± 0.03</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>Total ketone body (µmol/L)</td>
<td>47.3 ± 7.1</td>
<td>57.7 ± 10.7</td>
</tr>
<tr>
<td>Blood sugar (mmol/L)</td>
<td>5.19 ± 0.12</td>
<td>5.47 ± 0.18</td>
</tr>
<tr>
<td>Insulin (µmol/L)²</td>
<td>60.7 ± 5.2</td>
<td>78.4 ± 8.5</td>
</tr>
<tr>
<td>Leptin (mg/L)³</td>
<td>0.04 ± 0.00</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>PAI-1 (mg/L)</td>
<td>0.19 ± 0.03</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>Vitamin A (IU/L)⁴</td>
<td>1952 ± 89</td>
<td>2017 ± 98</td>
</tr>
<tr>
<td>Vitamin E (µmol/L)⁵,⁶</td>
<td>32.2 ± 1.3</td>
<td>30.3 ± 1.6</td>
</tr>
<tr>
<td>Malondialdehyde-modified LDL (µ/L)⁷</td>
<td>158 ± 11</td>
<td>134 ± 8</td>
</tr>
<tr>
<td>Glutamic-pyruvic transaminase (µkat/L)²</td>
<td>0.42 ± 0.04</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>Blood urea nitrogen (mmol/L)</td>
<td>4.95 ± 0.20</td>
<td>4.95 ± 0.22</td>
</tr>
<tr>
<td>Calcium (mmol/L)</td>
<td>5.05 ± 0.05</td>
<td>5.08 ± 0.03</td>
</tr>
<tr>
<td>Iron (µmol/L)</td>
<td>22.2 ± 1.4</td>
<td>22.4 ± 1.7</td>
</tr>
<tr>
<td>Inorganic phosphate (mmol/L)²</td>
<td>1.09 ± 0.04</td>
<td>1.21 ± 0.04</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>144 ± 1</td>
<td>143 ± 0</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>4.24 ± 0.06</td>
<td>4.37 ± 0.07</td>
</tr>
<tr>
<td>Chlorine (mmol/L)</td>
<td>106 ± 1</td>
<td>105 ± 0</td>
</tr>
</tbody>
</table>

¹ All values are x ± SEM. GTE, green tea extract; RLP cholesterol, remnant-like particles of cholesterol; PAI-1, plasminogen activator inhibitor 1. The initial values did not differ significantly between groups. Data from weeks 0 and 12 were compared by using two-factor repeated-measures ANOVA with time and group.
² Significant effect of time from week 0 to week 12, P < 0.05.
³ Significant time-by-group interaction, P < 0.05.

activities. Under these conditions, body weight, BMI, waist circumference, body fat mass, and SFA were significantly lower in the GTE group than in the control group. At week 12, the decrease in waist circumference, skinfold thickness, TFA, and SFA from the initial measurements was significantly greater in the GTE group than in the control group. The percentage difference in the changes in weight, BMI, waist circumference, body fat mass, skinfold thickness, TFA, and SFA between the GTE and control groups was 1.5%, 1.5%, 2.0%, 3.7%, 6.9%, 7.9%, and 7.5%, respectively. These results indicate that catechins not only promoted EE (18, 19) but also mildly reduced body fat in humans, as suggested by Chantre et al (21). Substances related to lipid metabolism and metal ions in the blood were also investigated, but there was no significant difference between the groups in any variable except vitamin E and MDA-LDL; nor were there any side effects.

Dulloo et al (18) observed that the addition of 200 nmol EGCg/mL increased thermogenesis in brown fat tissue of rat and that there was a synergistic effect between caffeine and EGCg. They also found that consumption of GTE (270 mg EGCg/d) increased EE and the oxidation of lipids in another human study (19). In those reports, they suggested that those effects were due to an inhibitory effect of catechins on the activity of catechol-O-methyltransferase (COMT), which is a catecholamine-degrading enzyme, and to an inhibitory effect of caffeine on phosphodiesterase that results in an increase in noradrenalin-induced thermogenesis and the maintenance of that increase (20). Other studies, however, reported that plasma EGCg concentrations were only ~4.5 nmol/mL after the intake of 525 mg EGCg/d in humans (38), and that the maximum in vitro inhibitory effect on the activity of COMT by 100 µmol flavonoids/L was 64% (39). These results indicate that the increase in thermogenesis and the reduction in body fat in humans cannot be completely explained by an inhibitory effect on COMT, and therefore another mechanism must be involved in the decrease in body fat induced by catechins.

Chemically modified, degenerated lipoproteins such as oxidative stress–induced MDA-LDL and 4-hydroxynonenal lysine–LDL are observed in atheroma in humans and rabbits (40). Holvoet et al (41) measured plasma MDA-LDL in humans and suggested both that an increase in plasma MDA-LDL can be used as a marker of unstable atherosclerotic cardiovascular disease and that blood MDA-LDL is an independent factor not correlated with LDL cholesterol. Previously, MDA-LDL was measured indirectly by using TBARS, and there are many reports that catechins prevent an increase in serum TBARS. In 2 reports, increases in BMI were significantly correlated with concentrations of TBARS (22) and malondialdehyde (23) in blood.
The current studies suggest that obesity might be related to an increase in lipid oxidizability. In addition, there is an interaction between nuclear factor-κ B and peroxisome proliferator–activated receptors (PPARs). PPARs are important transcription factors for lipid metabolism; for example, mRNA of β-oxidation enzymes is up-regulated by PPAR-α (42–45). Because nuclear factor-κ B is regulated by a redox regulatory system, it is possible that such a system also regulates body fat metabolism. This hypothesis led us to measure MDA-LDL by using an enzyme-linked immunosorbent assay and an anti–MDA-LDL antibody (ML25) to investigate the antioxidizing activity of ingested catechins and also to compare body fat variables. In the GTE group, the changes in concentrations of MDA-LDL were positively associated with the changes in body fat mass and TFA. These results suggest that the accumulation of body fat might be associated with an increase in lipid oxidizability and that a redox regulatory system might be involved individually in the body fat–and MDA-LDL–reducing effects of catechins. Further investigation is needed to clarify the relation between a redox regulatory system and the body fat–reducing mechanism.

Catechins reduce serum lipids by inhibiting small-intestine micelle formation in animals (46), and they limit the absorption of sugars by inhibiting α-glucosidase activity (47). There was no significant difference in serum lipids or blood sugar between the groups; however, the initial values might have been too low for any effects to be detected. Thus, the amount of catechins ingested in this study might not have been sufficient to inhibit micelle formation or α-glucosidase activity.

In conclusion, long-term consumption of beverages containing catechins inhibits the formation of oxidized lipids such as MDA-LDL, and this is a risk factor for developing arteriosclerosis. Moreover, catechin intake decreases body fat. These results suggest that catechins contribute to the prevention of and improvement in various lifestyle-related diseases, particularly obesity. These findings also suggest that regulation of a redox regulatory system might influence the accumulation of body fat.

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TN, YK, SS, and SM conducted the study. YK and SS contributed to data collection and database management. SM and TH assisted in the statistical analyses and contributed to data analyses. YH provided the samples, and TN designed and contributed to the writing of the manuscript. IT served as laboratory director, oversaw the writing of the manuscript, and was responsible for the diagnosis and recruitment of participants. None of the authors of this manuscript had any conflicts of interest.

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Duodenal ascorbate and ferric reductase in human iron deficiency1–3

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ABSTRACT

Background: The first step in iron absorption requires the reduction of ferric iron to ferrous iron, a change that is catalyzed by duodenal ferric reductase. Iron deficiency is associated with high iron absorption, high ferric reductase activity, and high duodenal ascorbate concentrations in experimental animals, but it is not known whether a relation between reductase and ascorbate is evident in humans. Objective: The objective of the study was to assess the relation between ferric reductase activity in human duodenal biopsy specimens and ascorbate concentrations in iron-replete and iron-deficient subjects. Design: Patients and control subjects were overnight-fasted adults presenting sequentially for upper gastrointestinal endoscopic investigation. Ferric reductase activity in duodenal biopsy specimens was assayed by using nitroblue tetrazolium. Ascorbate was assayed in duodenal biopsy specimens and plasma.

Results: Iron-deficient patients had significantly higher reductase activity (n = 6–9; P < 0.05) and duodenal (n = 20; P < 0.001) and plasma (n = 6; P < 0.001) ascorbate concentrations than did control subjects. Incubation of biopsy specimens with dehydroascorbate (to boost cellular ascorbate) increased reductase activity in the tissues that initially had normal activity (n = 9; P < 0.01) but inhibited reductase activity in the tissues that already had high reductase activity (n = 13; P < 0.001).

Conclusions: Iron deficiency in humans is associated with increased duodenal ascorbate concentrations. This finding suggests that increased reductase activity is partly due to an increase in this substrate for duodenal cytochrome b reductase 1.

KEY WORDS Iron absorption, iron nutrition, vitamin C

INTRODUCTION

Studies in both humans (1, 2) and mice (3–5) have implicated duodenal ferric reductase [catalyzed by duodenal cytochrome b (Dcytb) or cytochrome b reductase 1 (Cybrd1)] activity as an important factor in the regulation of intestinal iron absorption in both healthy subjects and those with altered iron metabolism. Duodenal ferric reductase activity is increased in persons with iron deficiency (1) and hemochromatosis (1, 2). We showed in mice that Dcytb activity is partly dependent on the concentration of its intracellular substrate, ascorbate (3, 6) and that concentrations of duodenal ascorbate are higher in mice with iron deficiency than in those that are iron replete (7). It follows from this that intracellular concentrations of ascorbate in the duodenal epithelium may be a factor in controlling intestinal iron absorption in humans. We therefore set out to investigate the alterations of ascorbate concentrations and ferric reductase activity in duodenal biopsy specimens from control subjects and patients with iron deficiency.

SUBJECTS AND METHODS

Subjects

Overnight-fasted adults (mixed group of males and females; age range: 25–91 y) presenting sequentially for upper gastrointestinal endoscopic investigation were the subjects for study. Duodenal biopsy specimens (n = 3 from second part of duodenum) were obtained during routine gastroscopy. One specimen was sent for routine histologic tests, and the other 2 specimens were used for 1 of 3 studies. In all patients, blood samples were taken for serum ferritin and hemoglobin assays.

Written informed consent was obtained from all subjects. Ethical approval for the study protocol was obtained from the ethics committees of St Thomas’ Hospital and King’s College Hospital.

Study design

In study 1, biopsy specimens were taken for immediate assay of reductase activity (reductase activity is labile on storage of samples) as described below. In study 2, biopsy specimens were homogenized in 5% ice-cold metaphosphoric acid by using a high-speed Ultra Turrax homogenizer (IAK Werke; Kanke & Kunkel BmVH & Co KG, Stauffen, Germany) and analyzed for ascorbate acid (AA) and dehydroascorbate acid (DHA) concentrations. Plasma was obtained by whole-blood centrifugation with the use of EDTA as an anticoagulant and assayed for AA and DHA concentrations. In studies 1 and 2, the iron status of each subject was not known to the investigators until all analyses were completed, at which point subjects were allocated to the iron-deficient (ferritin < 30 μg/L) or the control (ie, iron-replete;
Ascorbate assays

Ascorbate was assayed as described previously (7). The spectrophotometric determination was modified from an assay described by Kampfenkel et al (9) and based on the reduction of Fe$^{3+}$ to Fe$^{2+}$ by AA and on spectrophotometric detection of Fe$^{2+}$ complexed with ferrozine. DHA was reduced to AA by incubation of the samples with dithiothreitol. Excess dithiothreitol was removed with ethylmaleimide, and total AA was determined by the same procedure. The concentration of DHA was then quantified by densitometry (Student’s *t* test, *P* = 0.026).

**TABLE 2**

Hemoglobin and ascorbate concentrations in control and iron-deficient subject groups in study 2

<table>
<thead>
<tr>
<th>Subject group</th>
<th>Hemoglobin</th>
<th>Duodenal ascorbate</th>
<th>Plasma ascorbate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/L</td>
<td>µmol/g</td>
<td>µmol/L</td>
</tr>
<tr>
<td>Control (n = 23)</td>
<td>135 ± 15</td>
<td>13 ± 7</td>
<td>76 ± 12</td>
</tr>
<tr>
<td>Iron-deficient (n = 17)</td>
<td>111 ± 18</td>
<td>15 ± 7</td>
<td>76 ± 12</td>
</tr>
</tbody>
</table>

All results are expressed as means ± SDs. Differences between groups were analyzed by using paired or unpaired Student’s *t* test as appropriate (MICROSOFT EXCEL 2002; Microsoft Corp, Redmond, WA).

**RESULTS**

All subjects had normal results on duodenal histologic tests. The results of ferritin and hemoglobin assays in the subject groups for studies 1 and 2 are shown in Tables 1 and 2. Iron-deficient patients had significantly lower hemoglobin and serum ferritin concentrations (both: *P* < 0.001) than did iron-replete subjects in both studies. The values were similar in the corresponding groups in both studies.

Reductase activity was measured with NBT reduction (study 1) and was found to be higher in iron-deficient subjects than in control subjects (*Figure 1; *P* < 0.05). AA concentrations in both plasma and duodenum (study 2) were higher in duodenal biopsy specimens from subjects with iron deficiency than in those from iron-replete subjects (Table 2). DHA was below the detection limit (0.1 µmol/mg or 5 µmol/L) in most biopsy and plasma samples from both subject groups and overall did not differ significantly (*P* > 0.4) from zero in either group (data not shown).

In addition, we found (study 3) that, when reductase activity was low in biopsy specimens, the inclusion of DHA, which is taken up by cells and converted into AA (7, 11), tended to increase reductase activity (*Figure 2; *P* < 0.01). Paradoxically, DHA had an inhibitory effect on reductase activity in samples
Dcytb, ascorbate, is higher in persons with iron deficiency than particular, the concentration of the intracellular substrate for reductase protein, Dcytb; 1) by which stimulated, and that would result in reduced deposition of NBT-formazan there. We therefore hypothesize that, when AA concentrations exceed a threshold, mucous secretion can be enhanced by AA, namely, the enhancement of ferric reductase activity.

**REFERENCES**


**FIGURE 2.** Effect of incubation with dehydroascorbate (DHA) on reductase activity. Reductase activity was measured with nitroblue tetrazolium in 2 biopsy specimens from each patient: one in the presence of 10 mmol DHA/L (right column) and the other without added DHA. Data from patients with low activity (<0.04; white) was increased by DHA (P < 0.01), whereas activity from biopsy specimens with high reductase (>0.04; ■) was decreased by DHA (P < 0.001).

with higher initial activity (Figure 2; P < 0.001), which suggests a threshold for the effect.

**DISCUSSION**

The present data support the findings of Zoller et al (1) that duodenal ferric reductase activity [here measured with the chromogenic substrate NBT (1, 3, 7)] is higher in persons with iron deficiency than in those who are iron replete. Zoller et al showed that iron-deficient patients have significantly higher expression of Dcytb than do iron-replete patients (1). We have further investigated the basis for increased reductase activity and found that iron-deficient subjects have greater concentrations of the putative intracellular substrate for ferric reductase—namely, ascorbate—than do iron-replete subjects (6, 7). The present data also show that, when reductase activity is initially low, in vitro incubation of tissue with DHA leads to an increase in reductase activity. Incubation with DHA enhances intracellular AA concentrations (7, 11), which suggests that those concentrations can be limiting for reductase activity in human duodenum. It is interesting that, when reductase (and presumably endogenous AA) concentrations were initially high, incubation with DHA led to an inhibition in reductase activity. AA feeding has previously been reported to enhance mucous secretion by rat stomach, presumably by sparing glutathione and protecting cells from oxidative damage (12). Our own studies (not shown) in which biopsy specimens from mouse intestine were incubated with 1 mmol AA/L showed that such high concentrations of medium AA provoked mucous hypersecretion, which could prevent the access of NBT to the biopsy specimen’s surface and the deposition of NBT-formazan there. We therefore hypothesize that, when AA concentrations exceed a threshold, mucous secretion can be stimulated, and that would result in reduced deposition of NBT-formazan.

These findings provide an additional mechanism (ie, supplementing increases in the reductase protein, Dcytb; 1) by which human duodenal ferric reductase activity can be increased. In particular, the concentration of the intracellular substrate for Dcytb, ascorbate, is higher in persons with iron deficiency than in those who are iron replete. Other effects such as mucous secretion may, however, limit the increase in reductase, at least in in vitro studies. In vivo effects of AA on mucous secretion are likely governed by complex factors, including AA transport, oxygen concentrations, antioxidant defenses, and additional regulators of mucous secretion. Moreover, in vivo effects of AA on overall iron absorption will include ascorbate’s well-known iron-solubilizing and iron-reducing effects (13), which tend to counteract inhibitory effects due to increased mucous secretion. The finding that duodenal AA is higher in iron-deficient subjects than in those who are iron replete is in keeping with studies showing that iron concentrations generally are inversely related to tissue AA concentrations (14). However, epidemiologic studies found a positive correlation between dietary AA intake and iron stores (15). The relation between dietary AA intake and iron status has proved more complex than expected from studies with test meals (13, 16, 17). Whether dietary AA intake is the main regulator of ferric reductase activity requires further investigation, which will demand studies in which the subjects are strictly controlled for dietary AA intake as well as other factors that influence AA concentrations, such as smoking (18). The present findings provide an additional mechanism by which iron absorption can be enhanced by AA, namely, the enhancement of ferric reductase activity.

We are grateful to Richard P Thompson of St. Thomas’ Hospital, London, and the staffs of the endoscopy units of both St. Thomas’ and King’s College hospitals for help with provision of human samples.

BDA carried out ascorbate assays, assisted with study design, and collated data; ACYL and JB supplied biopsy specimens and collated data; KNT assisted with study design; and RJS carried out reductase assays, assisted with study design, and collated data. None of the authors had a conflict of interest.
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Differential effects of seasonality on preterm birth and intrauterine growth restriction in rural Africans

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ABSTRACT

Background: Low birth weight (LBW) can result from prematurity or intrauterine growth restriction (IUGR) and result in small-for-gestational-age (SGA) infants. Prematurity and IUGR may have different etiologies and consequences.

Objective: Our objective was to analyze seasonal patterns of prematurity and SGA in a rural African community and to compare them against variations in nutritional and ecologic variables that may provide insight into likely causative factors.

Design: Fourier series were used to compare the seasonality of prematurity (<37 wk) and SGA (<10 percentile of the reference standard) among 1916 live infants born over 26 y in 3 Gambian villages. The resultant patterns were compared against monthly variations in birth frequency, maternal energy status, maternal work, and malaria infections.

Results: The incidence of LBW was 13.3%, of prematurity was 12.3%, and of SGA was 25.1%. Prematurity and SGA showed divergent patterns of seasonality. Incidence of SGA was highest at the end of the annual hungry season, from August to December (peaking in November at 30.6%), with a nadir of 12.9% in June. Rates of SGA varied inversely with maternal weight changes. This pattern was not seen for rates of prematurity, which showed 2 peaks—in July (17.2%) and October (13.9%). The lowest proportion of preterm births occurred in February (5.1%). The peaks in prematurity closely paralleled increases in agricultural labor (July) and malaria infections (October).

Conclusion: We conclude that a reduction in LBW in such communities may require multiple interventions because of the variety of precipitating factors. Am J Clin Nutr 2005;81:134–9.

KEY WORDS Low birth weight, prematurity, intrauterine growth restriction, season, developing countries, pregnancy, gestational age, birth weight

INTRODUCTION

Birth weight is the product of the length of gestation and the rate of fetal growth. Low birth weight (LBW), defined as a birth weight <2500 g, could be the product of prematurity (<37 wk), intrauterine growth restriction (IUGR), or both. Because of the difficulty of diagnosing pathologic restraint or growth retardation in the fetus, weight-for-gestational age at birth is often used as a proxy measure for IUGR. Small-for-gestational-age (SGA) is defined as a birth weight below the 10th percentile for gestational age on the basis of a sex-specific reference standard (1, 2). It is important to distinguish between the 2 types of LBW because of differences in the consequences to the infant in terms of mortality and morbidity (3–5). Furthermore, the etiologies for both types of LBW are also heterogeneous (6–8).

Seasonality of LBW is a well-known phenomenon in developing countries (9–11). This has been attributed to the seasonal deterioration of nutritional status due to food shortages and an increase in agricultural labor that is often coincident with seasonal epidemics of infectious and parasitic diseases (12–14). There have, however, been no studies separating LBW seasonality according to prematurity or IUGR.

The West Kiang District in the Gambia has a seasonal agricultural system that revolves around an annual rainy season from July to November. Studies that have looked at the seasonality of weight gain among pregnant mothers in rural areas of the Gambia show that weight gain during the rainy season is 400–500 g/mo less than weight gain during the dry season (15–17). Birth weight has also been shown to be almost 90 g lower in the rainy season than in the dry season (17–19), with a greater difference from peak to trough.

The villages of Keneba, Manduar, and Kantong Kunda in the West Kiang District have been part of longitudinal demographic and health surveys since 1949. Data on maternal pregnancies, birth anthropometric measures, and gestational ages have been collected since 1978. The availability of these data enabled us to separately analyze the seasonality of prematurity and IUGR and to compare them against the external environmental factors that may play a precipitating role.

SUBJECTS AND METHODS

This was a retrospective cohort study of all live births in 3 subsistence-farming villages of the West Kiang District in the

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Gambia from 1976 to the present. The West Kiang District is an area of savannah and farmland, roughly 750 km², bounded on 3 sides by the River Gambia and its tributaries. The villages are rural and are ≈100 km from Banjul, the capital. There is a seasonal agricultural system that revolves around an annual rainy season, which occurs from July to November.

Regular ante- and postnatal care has been provided by resident midwives to all women of childbearing age since the mid-1970s. Birth weights were first recorded from the 3 villages in 1976. Birth weights were recorded to the nearest 10 g with a Salter spring balance and tared sling (Salter Industrial Measurements Ltd, West Bromwich, United Kingdom), which was calibrated regularly. LBW was defined as weight <2500 g. Measurement of gestational age was introduced in 1978, which was assessed by using the physical and neurologic scoring system validated by Dubowitz et al (20) by medical doctors within 5 d of delivery. Prematurity was defined as a gestational age <37 completed wk. SGA was defined as a birth weight less than the 10th percentile of gestational age based on the reference standard by Williams et al (1, 2).

Since regular antenatal clinics were started, pregnant women from the 3 villages were seen every 4 wk until the 28th wk and then fortnightly until term. Thick and thin blood smears for malaria were routine during these visits. The percentage of positive malaria smears among pregnant or lactating mothers during routine follow-up per calendar month were taken from records of these consults. There were a total of 43 385 visits and 3276 cases of malaria among 940 mothers from 1978 to 2003.

Regular anthropometric measurements from an earlier study of women of reproductive age from the same 3 villages from 1978 to 1988 were used to define the monthly change in body weight (15). Over this 11-y period, 13 833 weights from 529 women were collected—an average of 26 weights per woman. Within-subject regression was used to adjust for stage of pregnancy or lactation and year of measurement. The mean (±SD) annual weight was 52.8 ± 7.8 kg.

Maternal workload measured as the mean duration (h/d) in which pregnant women were active per calendar month was taken from a previous study of 73 pregnant women in Keneba from 1978 to 1979 with the use of a combination of activity diaries and 24-h activity recall (21). The amount of activity among pregnant women was markedly seasonal. The women were active 50% of a 15-h day from January to April, but this activity increased dramatically at the start of the farming season in June and July to 83%. Total energy expenditure was at a minimum at 9.6 MJ/d from January to March and at a maximum at 11.3 MJ/d from July to October.

Although the women’s weight data, physical activity, and malaria infection rates were not contemporaneous with the 1916 deliveries we describe, we considered these data acceptable because of the consistent seasonality that we observed over many years and because the 1916 births were themselves spread over 26 y.

There were 2 previous supplementation trials, which included ≥1 of the 3 villages (18, 19). The first-order effects of supplementation (fitted with the use of a single indicator variable) on preterm births and on SGA were not significant. The seasonality of either the preterm births or SGA was not significantly modified by supplementation when season was modeled by a simple binary variable indicating the hungry season (July to November). Thus, we ignored supplementation in the rest of the analysis.

The recording of births and deaths in these villages predated the formation of the Joint Gambian Government/Medical Research Council Gambia Ethics Committee. Approval for the continuation of the demographic surveillance was granted when the committee was formed in 1981.

Statistics

Fourier series are the natural mathematical models for seasonality. They are smooth linear functions whose terms are approximately orthogonal to one another and are inherently cyclic: the smoothness carries over from December to January. They also offer flexibility in the dimensionality of the fitted seasonal effect: truncating the higher-order terms of the series removes higher-frequency noise. If we use the first $p$ pairs of terms of the series, the seasonal component of the linear predictor would be as follows:

$$
\sum_{i=1}^{p} \beta_i \sin(r \theta_i) + \gamma_i \cos(r \theta_i)
$$

where angle $\theta_i$ is the point in the annual cycle that the $i$th child’s birthday occurred. Denoting the number of days between 1 January 1976 and the $i$th child’s birthday as $D_i$, we calculated this angle in radians:

$$
\theta_i = 2\pi(D_i \mod 365.25)/365.25
$$

In these data we found that only the first 3 pairs of terms (F3 model) were significant in these data.

We then investigated the seasonality of 2 binary outcome variables—SGA and prematurity—separately (univariate analysis) using logistic regression. Then, to compare the seasonality of the 2 outcomes simultaneously (bivariate analysis), we applied “seemingly unrelated biprobit regression” analysis (22). This method allowed us to fit separate models for SGA and prematurity simultaneously and to compare their goodness-of-fit when the same or different parameters were used in the seasonal component. We used the likelihood ratio test to compare models that fitted a separate set of underlying seasonality parameters for SGA and prematurity with models in which the underlying seasonality parameters were constrained to be identical for both outcomes. All analyses were performed with STATA 8 (Stata Corp, College Station, TX).

Results

There were 2977 live births in Keneba, Manduar, and Kantong Kunda from 1976 to 2003. Of these live births, 2472 (83.0%) had recorded birth weights. Of the 2550 births since 1978, 1918 (75.2%) had recorded gestational ages. Those without gestational ages were not significantly different from those with recorded gestational ages in terms of mean birth weight (difference in means = 17 g; 95% CI: −25, 59 g; $P = 0.433$), sex (Pearson’s $\chi^2 = 2.96$ on 1 df, $P = 0.085$), and month of birth (Pearson’s $\chi^2 = 15.4$ on 11 df, $P = 0.166$). Missing gestational ages were usually due to logistical problems (eg, doctor indisposed, no transport to village, and late information) and were more likely in the earlier years when the records were less complete. There were 2 infants with gestational ages but whose birth weights were not recorded. In all, there were 1916 infants with both birth weight and gestational age. The exact date of birth was known for all.
The incidence of LBW in this population was 13.3%, of prematurity was 12.3%, and of SGA was 25.1%. The relation between LBW, prematurity, and SGA is shown in Figure 1. Note that the 10th percentile of the reference standards falls almost exactly across the intersect of 2500 g and 37 wk gestation but does generate a small group of 15 term infants (12 at 37 completed weeks of gestation and 1 each at 38, 39, and 41 wk) with birth weights between 2100 and 2500 g. They were a paradoxical cluster of LBW infants who were born at term and were appropriate-for-gestational age. The month-by-month percentage of SGA infants is shown in Figure 2. The percentage of SGA infants was highest from August to December (peaking in November at 30.6%), with a gradual decrease in the percentage of SGA infants until the nadir in June at 12.9%. The percentage of preterm infants showed 2 peaks: one in July (17.2%) and one in October (13.9%). The lowest percentage of preterm births occurred in February (5.1%), which was followed by a gradual increase in the percentage of preterm infants until the peak in July. Logistic regression applied separately to each variable, fitting the first 3 pairs of Fourier terms, showed that both outcomes were significantly dependent on the season of birth (SGA: likelihood ratio $\chi^2 = 24.73$ on 6 df, $P = 0.0004$; preterm: likelihood ratio $\chi^2 = 20.07$ on 6 df, $P = 0.0027$). Use of a monthly 12-level stratification of the year did not result in a significant improvement over the F3 model of seasonality for either outcome (SGA: likelihood ratio $\chi^2 = 10.54$ on 11 df, $P = 0.48$; preterm: likelihood ratio $\chi^2 = 9.35$ on 11 df, $P = 0.59$). To test whether these prima facie patterns of seasonality of SGA and preterm deliveries (the unconstrained fitted curves in Figure 2) were significantly different, both outcomes were fitted simultaneously to the F3 model of seasonality by using seemingly unrelated biprobit regression. The regressions gave a significantly poorer fit to the data when the coefficients of Fourier variables are constrained to be identical than when a separate set of seasonality parameters was used for each outcome (likelihood ratio $\chi^2 = 23.06$ on 6 df, $P = 0.0008$), which confirmed that the patterns of seasonality are different for SGA and preterm births. The temporal similarity between rates of SGA and the annual variation in birth frequency and alterations in maternal energy balance are shown in Figure 3 and Figure 4. The number of births per month among all the births from the 3 study villages from 1978 to 2003 is shown in Figure 3. There was a marked fluctuation in birth frequency. The lowest birth rates were from April to July, coinciding with the lowest rates of SGA. Seasonal changes in maternal weight taken from a previous study by Cole (15) for women of reproductive age from the same population are shown in Figure 4. The gap between food intake and the energy costs of physical activity that occurs at the onset of the rainy season, because of dwindling food stores coincident with increased agricultural labor, causes an annual cycle of weight loss and gain in these rural villages. During the hungry season, an average woman in this population loses $\approx 2.6$ kg, $\approx 5\%$ of her body weight, or 25% of body fat. This weight loss is equivalent to an energy deficit of 10–15%/d, which closely parallels the rate of SGA in the community with the lowest rates during the months with the heaviest body weights. Fluctuations in the rates of preterm births, with variations in maternal workload and malaria infections, are shown in Figure 5 and Figure 6, respectively. The percentage of preterm infants
born per month relative to the proportion of a 15-h d in which pregnant women from Keneba are active, as measured from a previous study by Roberts et al (21), is shown in Figure 5. Agricultural work increases in the area, which may start with clearing and manuring of fields, as early as April or May and intensifies to a peak in June and July when the first rains necessitate long hours of hoeing, planting, and weeding. This abrupt increase in workload from April to July is matched by an increase in the rate of preterm births during the same period. The percentage of pregnant or lactating mothers with positive findings on malaria smears, which were conducted during routine 2- to 4-weekly follow-ups per calendar month in the 3 study villages from 1978, is shown in Figure 6. The highest rates of parasitemia were seen late in the rainy season, from September to November. This was tracked by an increase in the rate of preterm births during these months.

DISCUSSION

The monthly percentages of prematurity and SGA showed 2 distinctly divergent patterns, which emphasized the different etiologies. Elsewhere we argued that the underlying seasonal factors affecting SGA and prematurity could be explained by a common synchronicity if we make the reasonable assumption that fetal growth retardation leading to SGA accumulates over the duration of gestation but that the factors precipitating early parturition act essentially instantaneously (AJ Fulford, unpublished observations, 2004). This does not, however, confirm that the causes definitely are synchronous nor does it imply that they are the same.

Inferences about the likely casual factors for SGA and prematurity can be drawn by evaluating the effects of other ecologic data on seasonality in this population. The following discussion considers the possible effects of selective conception, maternal energy balance, maternal workload, and malarial infections on SGA.

In the population studied, there was a clear seasonal variation in birth frequency (Figure 3). The nadir from April to July comes 9 mo after the annual hungry season in which weight loss occurs from July to September. This would fit with the Frisch hypothesis that the pituitary-gonadal reproductive axis is regulated by maternal energy flux (23, 24), but alternative explanations involving seasonal variation in coital frequency cannot be ruled out. If the former (ie, physiologic) explanation were true then it would imply that the infants born in the nadir of birth frequency represent a selected subgroup defined by greater maternal or embryonic fitness. In separate studies we are investigating possible “thrifty genes” that might confer a greater likelihood of conception or fetal survival during these times of especially harsh nutritional conditions. In the current context it is necessary to ask whether these factors might also be associated with differences in fetal growth or length of gestation. There is a tolerably close fit between the seasonality in birth frequency and SGA births, which is consistent with there being a higher proportion of “fitter” fetuses implanted when conception rates are at their lowest. However, alterations in substrate supply to the fetus might offer a more straightforward explanation.
There is a gap between food intake and the energy costs of physical activity that occurs at the onset of the rainy season because of dwindling food stores coincident with increased agricultural labor. This imbalance causes an annual cycle of weight loss and gain among women of reproductive age in these rural villages. The resulting variation in maternal weight reflects this energy balance. This fluctuation corresponded closely to rates of SGA, as shown in Figure 4. Rates of SGA get progressively lower as the harvest season progresses from February to June and are highest in the hungry season from August to December. This association supports findings from a systematic review of 13 randomized controlled trials involving 4665 women to treat or prevent IUGR (25). Balanced energy and protein supplementation (when the protein content of the supplement was <25% of the total energy content) was associated with moderate increases in maternal weight gain (difference in means: 17 g/wk; 95% CI: 5, 29 g/wk) and mean birth weight (difference in means: 25 g; 95% CI: 4, 55 g) and a considerable decrease in risk of SGA births (odds ratio: 0.64; 95% CI: 0.53, 0.78). A path analysis done by Susser (26) showed that the causal pathway between dietary intake and birth weight bypass maternal weight change outside famine conditions, which may partly explain the modest weight gain alongside a substantial decrease in SGA seen in the review. In our setting of intense and acute annual nutritional deprivation, this relation between maternal weight and SGA is more noticeable. Indeed, in the Gambian supplementation study (18), the high-energy food supplement had a relatively large effect on fetal growth and SGA.

The rate of prematurity did not follow this same trend. There is little evidence in the published literature of an association between maternal nutritional status and risk of premature delivery. Studies of gestational weight gain (27), food supplementation (25), and nutritional advice (28) failed to show conclusive evidence of relations between nutritional status in pregnancy and the resultant length of gestation.

The prematurity rate was highest in July. This corresponds with the increase in agricultural work in the area from April to July (Figure 5). In the pregnant women from these 3 rural villages, there was only a small decrease in total energy expenditure of ≈0.6 MJ/d from the 28th wk of gestation until 4 wk postpartum (29). Heavy work, especially agricultural work among women in developing countries, is a risk factor for prematurity (30–34). The fact that much of the work involves standing and bending may exacerbate this risk.

A recent study of sheep gestation showed that undernutrition in the periconceptional period can cause premature birth (35). Ewes who were undernourished from 60 d before to 30 d after conception, such that they lost 15% of their weight, gave birth after an average of 139 d of gestation as compared with 146 d in well-nourished ewes. In our study, the lowest maternal weights in October would closely correspond to the highest rates of prematurity (38–42). However, controlled trials of the effect on prematurity of chemoprophylaxis (43, 44) or of the use of bed nets (39, 45) during pregnancy have been few and have had conflicting results. No firm conclusion regarding an association can be drawn from the current study.

In the rural, subsistence-farming women in the Gambia, the agricultural system, which revolves around an annual rainy season, creates an “experiment of nature” with regular hungry and harvest seasons that result in yearly mobilization and redeposition of body fat stores (15). Coupled with meticulous record keeping throughout the years, the seasonal agricultural system provides a unique setting in which to study factors affecting reproductive outcomes. In summary, our data show that seasonal patterns of maternal weight change closely parallel the rate of SGA births in rural Gambia. This pattern is not seen in rates of prematurity that, for the July peak, more closely parallel increases in agricultural labor and possibly in periconceptional energy status and, for the October peak, might be related to malaria infection rates. Because many factors are likely involved in the seasonality of LBW and preterm births, there is a need for further study to understand this phenomenon and plan targeted strategies for implementation in various settings.

We are grateful to the villagers of Keneba, Manduara, and Kantong Kunda in West Kiang for their willing participation in the studies of the Medical Research Council, Gambia.

PR-S was responsible for the conception of the study, data interpretation, and manuscript preparation. AJF was responsible for the data analysis and interpretation and manuscript preparation. AMP assisted in the conception of the study, interpretation of results, and critical revision of the report. All authors contributed to the writing of the final manuscript. None of the authors had a conflict of interest.

REFERENCES


Growth of children at high risk of obesity during the first 6 y of life: implications for prevention\textsuperscript{1–3}

Robert I Berkowitz, Virginia A Stallings, Greg Maislin, and Albert J Stunkard

**ABSTRACT**

**Background:** The contribution of familial factors to adiposity in children is poorly understood.

**Objective:** The objective was to assess differences in growth in the first 6 y of life in children born to either overweight or lean mothers.

**Design:** The body size and composition of 33 children at high risk and 37 children at low risk of obesity on the basis of the mother’s overweight [body mass index (BMI; in kg/m\(^2\)) of 30.2 ± 4.2 and 19.5 ± 1.1, respectively] were measured repeatedly from 3 mo to 6 y of age at the Children’s Hospital of Philadelphia.

**Results:** At year 2, no significant differences in any measure were observed between the high- and low-risk groups. By year 4, weight, BMI, and lean body mass were greater in the high-risk than in the low-risk children. By year 6, weight was even greater in the high-risk than in the low-risk children (23.4 ± 6.4 compared with 20.4 ± 2.1 kg; \(P < 0.02\)), and, for the first time, fat mass was greater in the high-risk than in the low-risk children (6.7 ± 5.7 compared with 3.8 ± 1.2 kg; \(P < 0.02\)). Ten of 33 high-risk children exceeded the 85th percentile of BMI at year 6 compared with 1 of 37 low-risk children (odds ratio = 15.7). Accelerated weight gain was predicted by high-risk group status, greater weight at year 2, and lower family income.

**Conclusion:** Anthropometric measures were not significantly different between groups at year 2; weight and lean body mass were greater at years 4 and 6, and fat mass was greater at year 6 in high-risk children. *Am J Clin Nutr* 2005;81:140–6.

**KEY WORDS** Childhood, obesity, genetic influence, risk factors, body weight, fat mass, skinfold thickness

**INTRODUCTION**

The epidemic of childhood obesity (1, 2) is regularly attributed to the toxic environment of readily available, calorically dense food and drink (3–5). How does this environment interact with the genetic vulnerability to determine who becomes obese (6–9)? What can this interaction tell us about who becomes obese and at what age they become obese? Answers to these questions could be of great value in preventing childhood obesity, and longitudinal studies of child development are well suited to answering these questions.

There have been few longitudinal studies of obesity in children, and only 2 have studied children at high risk of obesity. One study included 12 infants of overweight mothers and 6 infants of lean mothers (10). The other study involved 12 children with at least one obese parent and 13 children of normal-weight parents (11). Each study reported the development of overweight in the offspring of overweight mothers. Another longitudinal study (of a random population) found significant correlations between the body mass index (BMI) of children and that of their parents (12). These findings persuaded us to undertake a study of the growth of children of obese parents.

This study was a longitudinal investigation of the growth from birth to 6 y of age of 70 children, 33 of whom had overweight mothers and 37 of whom had lean mothers. Risk group was defined by prepregnancy maternal BMI (in kg/m\(^2\)): greater than the 66th percentile or less than the 33rd percentile for their age group (13, 14). During the first 2 y, the size and growth of children in the high- and low-risk groups were almost identical, and there was no relation between maternal and offspring body weights (14). This report describes the relation of risk group to the development of body size and body fat for this cohort from years 2 through 6 of life. A companion report will describe the social and behavioral influences on the somatic developments described below.

**SUBJECTS AND METHODS**

**Subjects**

The study began with 78 infants recruited at 3 mo: the present analyses were conducted on the 70 subjects who remained in the study by 6 y of age. Seven high-risk children and one low-risk child were lost to follow-up.

Mothers of the high-risk subjects had a prepregnancy BMI of 30.3 ± 4.2. The BMI of the mothers of the low-risk subjects was 19.5 ± 1.1 (\(P < 0.001\)). The 33 members of the high-risk sample included 16 boys, and the 37 members of the low-risk sample included 18 boys.

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Half of the overweight mothers had overweight husbands, whereas one-quarter of the lean mothers had an overweight husband. The BMI of the husbands of overweight mothers (27.1 ± 4.0) was much lower than that of their wives (30.3 ± 4.2; \( P < 0.001 \)) (Table 1). The study was confined to whites, because nonwhite children have different growth patterns (15–18). All subjects were born without complications after an uneventful, full-term pregnancy and uncomplicated delivery. Further details of study recruitment and design were reported previously (13, 14).

### Body size and composition

The subjects were examined every 3 mo during the first year, every 6 mo through year 4, and yearly thereafter. Measurements during the first 2 y were described previously (13, 14). From year 2, height was measured in triplicate in the laboratory with a stadiometer (Holtain, Crymych, United Kingdom) and weight in triplicate with a digital scale (model 6002; Sacletronix, Carol Strea, IL). Home visits used a Shorr Infant/Child Portable Measuring Board and a portable scale (model 5600; Sacletronix). Skinfold thicknesses were measured with a Holtain Skinfold Caliper at the biceps, triceps, subscapular, and suprailiac sites; waist circumference was measured at this time. All measurements were obtained by trained assistants whose techniques were standardized (19). Fat mass, percentage fat, and lean body mass (LBM) were determined at ages 3, 12, and 24 mo by total-body electrical conductivity as described previously (13, 14), and by dual-energy X-ray absorptiometry (model 2DR 2000; Hologic Inc, Bedford MA) at years 4, 5, and 6. Information about the parents included age, height, weight, BMI, and measures of socioeconomic status.

Childhood overweight was defined as a BMI at or above the 85th percentile for age and sex according to the National Center for Health Statistics/Centers for Disease Control and Prevention growth charts (20). At 6 y of age, the BMI threshold for girls was 17.0 and for boys was 17.1. Written informed consent was obtained from the parents. The protocol was approved by the institutional review boards of the University of Pennsylvania and The Children’s Hospital of Philadelphia. The study was conducted in the Nutrition and Growth Laboratory and the General Clinical Research Center of The Children’s Hospital of Philadelphia.

### Statistical analysis

Group means were compared with the use of \( t \) tests for groups with unequal variance. The significance of risk group differences in rates of overweight at years 4 and 6 was determined with Fisher’s exact tests. The odds ratio for obesity comparing the high- and low-risk groups was determined together with 95%
exact CIs (21). Individual variation in growth curves was characterized by graphing individual child profiles of measures of weight over time and summarizing these through the use of nonlinear mixed-model regression analyses. Specifically, a 2-stage regression algorithm used restricted maximum-likelihood estimation to estimate nonlinear growth parameters and thereby generate individual growth curves. Additional details of this method are included in the technical appendix. Analyses of covariance were then used to compare changes in growth as a function of obesity risk group, sex, income, and weight at year 2.

Graphic analyses were performed to examine how risk group differences emerged over time with the use of Glass’s effect size (the difference between population means divided by the SD of the population control group) (22). Effect sizes were estimated and displayed longitudinally from month 3 to year 6 for body size and composition. Effect sizes vary from small (0.2–0.4) and medium (0.4–0.8) to large (≥0.8). Changes in weight, height, weight z scores, BMI, skinfold thicknesses, and fat mass for the 3 groups were plotted: 1) high-risk subjects with values greater than or equal to the 85th percentile at year 6; 2) high-risk subjects with values less than the 85th percentile at year 6; and 3) low-risk subjects.

RESULTS

The differences between risk groups by Glass’s effect size estimates for 7 measures of growth up to year 6 are shown in Figure 1 (22). Large increases in effect size from years 2 to 6 for weight, BMI, sum of 4 skinfold thicknesses, percentage body fat are evident, as is a very high value of 2.4 for fat mass. The effect size for height was negligible.

The values at years 4 and 6, from which Figure 1 was constructed, are shown in Table 1. At year 2, there were no significant differences in weight, BMI, the sum of 4 skinfold thicknesses, waist circumferences, LBM, percentage body fat, and fat mass between the high- and low-risk children (14). By year 4, weight, BMI, LBM, and waist circumference were greater in the high-risk children than in the low-risk children (P < 0.03 for all). By year 6, the already greater weight, BMI, LBM, and waist circumference of the high-risk children increased more than that of the low-risk children, as did all skinfold thicknesses. For the first time, fat mass in the high-risk children (6.7 ± 5.7 kg) was significantly greater than that in the low-risk children (3.8 ± 1.2 kg; P = 0.02), as was percentage body fat (24.7 ± 11.8 compared with 18.8 ± 4.5; P = 0.03).

The high-risk children exceeded the 85th percentile of BMI more often than did the low-risk children. At 6 y of age, 10 (30%) of the high-risk and 1 (3%) of the low-risk children had BMI values above the 85th percentile (P = 0.01). Seven and 6 children in the high-risk group exceeded the 90th and 95th percentiles, respectively; none of the children in the low-risk group did (Table 2). The odds ratio (exact 95% CI) for overweight was 15.7 (95% CI: 1.9, 697.6) for a comparison of high-risk with low-risk children, with no evidence of sex differences.

The mixed-model regression that best fit the weight data contained substantial nonlinearity (θ = 2.4, see Appendix A) consistent with the accelerating weight gains of some high-risk children. Among high-risk children, the mean (±SD) for weight (nonlinear slope) was 24.0 ± 11.3 (range: 13.7–59.5). Among low-risk children it was 18.5 ± 3.6 (range: 12.2–26.0). Both the SDs (F = 10.2, df = 32, 36, P < 0.0001) and the mean values (t tests for groups with unequal variance = 2.71, df = 37.6, P = 0.01) differed between risk groups. Weight at year 2 did not influence the comparison of high- with low-risk nonlinear slopes of weight over time and thus was not a confounding influence.

In the analysis of covariance regression model, the difference in acceleration of weight gain between the high- and low-risk
groups was significant (adjusted difference in means = 0.005, SE = 0.05, P = 0.017). Low family income was an independent contributor to accelerated weight gain at a magnitude similar to that for the effect of risk group (adjusted difference in means = 0.005, SE = 0.05, P = 0.064). Increased baseline year 2 weight was associated (P = 0.006) with accelerated weight gains from month 3 to year 6 after control for risk group, sex, and family income. Each 1-kg increase in body weight at 2 y was associated with an additional weight increase in the nonlinear slope of 0.0024 (SE = 0.00084) kg/mo through year 6. Paternal weight had no significant effect on outcome in either analysis.

The results of the nonlinear mixed-model analysis comparing risk groups for weight, sum of 4 skinfold thicknesses, fat mass, percentage fat, BMI, weight z score, and BMI z score are shown in Table 3; all except percentage fat and fat mass were statistically significant.

The above analyses showed large differences in growth between the high- and low-risk subjects. Even greater differences were present within the high-risk group, which comprised subgroups: one overweight group and one normal-weight group. These differences between the high- and low-risk groups were due to the presence, within the high-risk group, of 10 overweight subjects. Differences in the distribution of high- and low-risk subjects between years 2 and 6 for the individual slopes of weight, sum of 4 skinfold thicknesses, and fat mass are shown in Figure 2. For each measure, the high-risk group had a number of subjects who had far greater increases in each measure than did other subjects. By contrast, there was little variability within the low-risk group.

Subjects were then divided into 3 groups: high-risk overweight, high-risk normal-weight, and low-risk groups. The BMI of the mothers of the high-risk overweight children (32.3 ± 4.6) surprisingly did not differ significantly from that of the high-risk normal-weight children (29.7 ± 4.0). The mean growth trajectories of the 3 groups are plotted in Figure 3, and a post hoc statistical analysis of differences was conducted. A striking increase from month 3 to year 6 was observed in the high-risk overweight group for 5 measures of adiposity: weight, weight z scores, BMI, sum of 4 skinfold thicknesses, and fat mass; there was no significant difference in height between risk groups. The panels contain a vertical line at the ages at which the high-risk overweight group began to differ (P < 0.05) from the other 2 groups, from 2.5 y for BMI to 4.0 y for fat mass. In contrast with the high-risk overweight group, the high-risk normal-weight and low-risk groups showed no evidence of increasing adiposity or any difference between them. Height did not differ significantly between the groups at any time.

Among the high-risk overweight group, BMI increased at 2.5 y of age (Figure 3D). Rolland-Cachera et al (personal communication, 2003) identified this increase in these data as an example of “adiposity rebound,” which she proposed as a predictor of obesity (23). By contrast, the 2 normal-weight groups showed no evidence of adiposity rebound during the 6 y of observation.

**DISCUSSION**

This study has traced the dramatic differences in growth of children at high or low risk of obesity as conferred by maternal overweight or leanness. At 2 y of age, the size and body composition of groups at high and low risk did not differ significantly. By year 4, the weight, BMI, and LBM of the high-risk subjects were significantly greater than those of the low-risk subjects, but the groups did not yet differ significantly in fat mass. By 6 y of age, the high-risk group had increased its difference from the low-risk group in weight, BMI, and LBM, and fat mass (6.7 ± 5.7 kg) had become significantly greater than that of the low-risk group (3.8 ± 1.2 kg; P < 0.02).

Studies of high-risk populations are particularly well suited to the investigation of obesity, because of the ease of identifying persons with a strong genetic potential. Furthermore, they permit a more efficient study of the determinants of obesity than can a study of persons with a normal distribution of body weights. Genetic contributions to obesity in adult life are striking (7, 24), with heritability ranging as high as 70% in a study of twins reared apart (8). High heritability was indicated in this study by an odds ratio of 15.7 in a comparison of high-risk with low-risk children by 6 y of age. As in the present study, Cardon (25) found negligible heritability for weight up to age 2 y. He then found a major increase between 3 and 6 y of age, the same years during which we observed dramatic increases in measures of adiposity. He proposed that genes for body weight are not activated at the same age but at different ages, thereby contributing to the evolving pattern of weight gain during growth and development.

Strong evidence for the activation of genes for body weight at different years is provided in this study by the 30% of high-risk children who became overweight by 6 y of age. Other children, also at high risk, were still no heavier than the low-risk children. Whatever their genetic potential for obesity, it was still not expressed.

As in previous longitudinal studies (26, 27), prior weight was a strong predictor of current weight. In this study, addition of paternal overweight made no contribution to the prediction of body weight in the children, probably because of the limited extent of the fathers’ overweight in the high-risk group (BMI = 27.1 ± 4.0).

The present study had limitations, including its relatively small sample size, which reduced the possible achievement of

**TABLE 2**
Distribution of BMI percentiles by risk group at year 6

<table>
<thead>
<tr>
<th>Percentile</th>
<th>High-risk group (n = 33)</th>
<th>Low-risk group (n = 37)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>85th</td>
<td>10 (30)</td>
<td>1 (3)</td>
<td>0.002</td>
</tr>
<tr>
<td>90th</td>
<td>7 (21)</td>
<td>0 (0.0)</td>
<td>0.004</td>
</tr>
<tr>
<td>95th</td>
<td>6 (18)</td>
<td>0 (0.0)</td>
<td>0.008</td>
</tr>
</tbody>
</table>

* Computed by Fisher’s exact test.

**TABLE 3**
Summary of results from the nonlinear mixed models: comparison of the high- and low-risk groups

<table>
<thead>
<tr>
<th>Factor</th>
<th>Estimated β</th>
<th>SE (β)</th>
<th>P</th>
<th>Partial R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>4.519</td>
<td>1.837</td>
<td>0.017</td>
<td>0.086</td>
</tr>
<tr>
<td>Sum of 4 skinfold thicknesses</td>
<td>6.316</td>
<td>2.495</td>
<td>0.014</td>
<td>0.094</td>
</tr>
<tr>
<td>Fat mass</td>
<td>0.265</td>
<td>0.142</td>
<td>0.068</td>
<td>0.063</td>
</tr>
<tr>
<td>Percentage body fat</td>
<td>112.298</td>
<td>77.410</td>
<td>0.153</td>
<td>0.004</td>
</tr>
<tr>
<td>BMI</td>
<td>24.467</td>
<td>8.217</td>
<td>0.005</td>
<td>0.122</td>
</tr>
<tr>
<td>Weight z score</td>
<td>10.858</td>
<td>3.990</td>
<td>0.008</td>
<td>0.104</td>
</tr>
<tr>
<td>BMI z score</td>
<td>192.491</td>
<td>68.164</td>
<td>0.007</td>
<td>0.119</td>
</tr>
</tbody>
</table>

* Time was defined in months in all regression models. For presentation purposes, the dependent variables in the regression models were multiplied by 1000 to eliminate leading zeros in the estimated slope coefficients.
statistical significance for between-group differences for some measures, such as weight gain by sex and the influence of paternal weight.

Note that in the present study, risk group was defined by maternal weight, which includes both genetic and environmental influences. Powerful genetic influences on BMI in adult life have been shown in both twin and adoption studies, and, in adult life, there is no evidence of childhood environmental influences (8, 28). Thus, the heritability of adult twins who had been reared apart did not differ significantly from that of twins reared together (8). However, studies of twins in adolescence showed significant shared environmental influences (28). Adoption studies found similar relations. There was no correlation between the BMI of adoptive parents and the children they had raised when the latter were 40 y of age (29). When the children were living with their adoptive parents during childhood, however, there was a statistically significant relation between their BMIs (30). We were unable to specify the amount of the variance due to genetic and common family environmental influences in predicting adiposity in the high-risk group in the present study. However, one environmental influence was clearly established: family income. It showed the same inverse relation with body weight that is found among adults (31). The parents in this study exerted this influence on their children.

FIGURE 2. Distribution of individual slopes for weight, sum of 4 skinfold thicknesses, and fat mass in the high-risk (n = 33) and low-risk (n = 37) subjects between years 2 and 6.
The low-risk group was featured in this report largely for comparison with the high-risk group; however, the experience of the low-risk group is important in its own right. Subjected to the same overall environmental influence as the high-risk group (and the population as a whole), only 1 of the 37 low-risk children exceeded the 85th percentile for age and none exceeded the 90th percentile. Genetic influences can evidently prevent as well as predispose to obesity.

This demonstration of the powerful effect of maternal overweight on the development of adiposity in childhood has important implications for prevention. It confirms, and extends, the findings of earlier reports of the influence of overweight parents on the development of obesity in their children (10–12, 32). It points to a potentially critical measure in the effort to prevent obesity in childhood. This measure is the identification of children at high risk of obesity, which encourages a focus of attention on them. Such focus allows intense effort directed to a relatively small, critical population, obviating the expense of large programs directed toward all children, few, if any, of whom could benefit.

The present study also indicates the optimal age for initiation of efforts at prevention. It is suggested by the sequence of developments in which increased body weight at age 4 y followed by increased body fat at age 6 y. Thus, efforts at prevention may be begun by age 4 y for the overweight children of overweight mothers. It is not necessary to wait for the appearance of increasing adiposity at 6 y in these already overweight children.

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FIGURE 3. Changes in measures of adiposity in the high-risk overweight (n = 10), high-risk normal-weight (n = 23), and low-risk (n = 37) groups. The vertical line in each panel depicts the age at which the high-risk overweight group differed significantly (P < 0.05) from the other 2 groups. A: By 3.5 y, weight was significantly greater in the high-risk overweight group than in the high-risk normal-weight and low-risk groups. B: The development of height was strikingly similar in the 3 groups. C: Weight z scores differed significantly between groups by age 3 y. D: BMI increased and decreased from month 3 to year 2 as expected. The decrease in BMI at 2 y was an artifact of the change in measurement from length to height at this age. Thereafter, the BMI of the high-risk overweight group increased to 17 at 2.5 y, which reflected “adiposity rebound,” whereas the BMI of the other 2 groups remained at 16. E: The sum of 4 skinfold thicknesses in the high-risk overweight group became significantly greater than that in the other 2 groups by age 3 y. F: Fat mass was significantly greater in the high-risk overweight group (13.0 kg) than in the other 2 groups (2.8 kg).
statistical analysis. AIS, RIB, and VAS were responsible for the critical revision of the manuscript for important intellectual content; administrative, technical, or material support; and supervision of the study. None of the authors had any conflict of interest.

REFERENCES
Congenital heart defects and abnormal maternal biomarkers of methionine and homocysteine metabolism\(^{1-4}\)

*Charlotte A Hobbs, Mario A Cleves, Stepan Melnyk, Weizhi Zhao, and S Jill James*

**ABSTRACT**

**Background:** It is well established that folic acid prevents neural tube defects. Although the mechanisms remain unclear, multivitamins containing folic acid may also protect against other birth defects, including congenital heart defects.

**Objective:** Our goal was to establish a maternal metabolic risk profile for nonsyndromic congenital heart defects that would enhance current preventive strategies.

**Design:** Using a case-control design, we measured biomarkers of the folate-dependent methionine and homocysteine pathway among a population-based sample of women whose pregnancies were affected by congenital heart defects (224 case subjects) or unaffected by any birth defect (90 control subjects). Plasma concentrations of folic acid, homocysteine, methionine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), vitamin B-12, and adenosine were compared, with control for lifestyle and sociodemographic variables.

**Results:** After covariate adjustment, case subjects had higher mean concentrations of homocysteine (\(P < 0.001\)) and SAH (\(P < 0.001\)) and lower mean concentrations of methionine (\(P = 0.019\)) and SAM (\(P = 0.014\)) than did control subjects. Vitamin B-12, folic acid, and adenosine concentrations did not differ significantly between case and control subjects. Homocysteine, SAH, and methionine were identified as the most important biomarkers predictive of case or control status.

**Conclusions:** The basis for the observed abnormal metabolic profile among women whose pregnancies were affected by congenital heart defects cannot be determined without further analysis of relevant genetic and environmental factors. Nevertheless, a metabolic profile that is predictive of congenital heart defect risk would help to refine current nutritional intervention strategies to reduce risk and may provide mechanistic clues for further experimental studies. *Am J Clin Nutr* 2005;81:147–53.

**KEY WORDS** Birth defects, congenital heart defects, risk, methionine, homocysteine, folate

**INTRODUCTION**

Congenital heart defects occur among \(\approx 11\) per 1000 live births in the United States (1) and are substantially more prevalent among stillbirths and miscarriages (2). Annually, heart defects account for \(\approx 6000\) total deaths and one-tenth of infant deaths (1). Only \(\approx 15\%\) of heart defects can be attributed to a known cause (1). The remaining cases are thought to result from complex interactions involving environmental exposures, maternal lifestyle factors, and genetic susceptibilities.
hydrolisis of SAH. Thus, elevated homocysteine concentrations cause SAH to accumulate (20). Increased SAH is a potent product inhibitor of cellular methyltransferases, which during organogenesis can alter gene expression, cell differentiation, and apoptosis (21, 22).

The goal of our study was to determine whether biomarkers of the folic acid pathway, including homocysteine and methionine metabolism are altered among nonpregnant women who have had a pregnancy affected by congenital heart defects compared with women without such a history. This may provide new insights into mechanisms that confer an increased risk of having pregnancies affected by congenital heart defects.

**SUBJECTS AND METHODS**

The protocol and provisions for informed consent were reviewed and approved by the Institutional Review Board at the University of Arkansas for Medical Sciences. Case subjects were identified and ascertained through the Arkansas Reproductive Health Monitoring System, a statewide birth defects registry. Case subjects were viewed and approved by the Institutional Review Board at the University of Arkansas for Medical Sciences. Case or control subjects who were pregnant at the time of the blood draw were excluded; only nonsyndromic congenital heart defects were included in this study.

Control subjects were Arkansas residents who had live births that were unaffected by any birth defect and were English or Spanish speaking. They were randomly chosen from all birth certificates registered at the Arkansas Department of Health, with birth dates between June 1998 and February 2003. Case or control subjects who were pregnant at the time of the blood draw or on antiepileptic medications were not eligible for the study.

After determining eligibility for case or control subjects, a research nurse contacted the subjects by mail and telephone, describing this local study. During scheduled home visits, the nurse obtained written informed consent and a blood sample by routine venipuncture.

**Covariates**

The maternal educational level and household income were reported during a structured computer-assisted telephone interview that was specifically designed for an ongoing multisite case-control study, the NBDPS). Further details about the NBDPS were published previously (23). At the time of the home visit, a Block Abbreviated Food-Frequency Questionnaire (24) was administered, and information about the current use of multivitamins, cigarettes, and alcohol and about caffeine intake was obtained.

**Sample preparation and biomarker measurement**

Blood samples were collected into evacuated tubes containing EDTA, immediately chilled on ice, and centrifuged at 4000 \( g \) for 10 min at 4 °C. Aliquots of the plasma layer were transferred into cryostat tubes and stored at −80 °C until analysis. Aliquots were thawed for extraction and HPLC quantification. Plasma folic acid and vitamin B-12 concentrations were measured by using Quanaphase II radioimmunoassay kit from Bio-Rad Laboratories (Hercules, CA).

To determine total homocysteine and methionine, 50 \( \mu L \) freshly prepared 1.43 mol/L sodium borohydride solution containing 1.5 \( \mu \)mol/L EDTA, 66 mmol/L NaOH, and 10 \( \mu L \) isomyl alcohol was added to 200 \( \mu L \) plasma to reduce sulfhydryl bonds. The samples were incubated at 40 °C on a shaker for 30 min. To precipitate proteins, 250 \( \mu L \) ice-cold 10% metaphosphoric acid was added and mixed well, and the sample was incubated for 10 min on ice. After centrifugation at 18 000 \( g \) for 15 min at 4 °C, the supernatant fluid was filtered through a 0.2- \( \mu m \) nylon membrane filter (PGC Scientific, Frederic, MD) and a 20- \( \mu L \) aliquot was injected into the HPLC system.

For determination of SAM, SAH, and adenosine, 100 \( \mu L \) 10% meta-phosphoric acid was added to 200 \( \mu L \) plasma to precipitate protein; the solution was mixed well and incubated on ice for 30 min. After centrifugation for 15 min at 18 000 \( g \) at 4 °C, supernatant fluids containing SAM and SAH were passed through a 0.2- \( \mu m \) nylon membrane filter, and 20 \( \mu L \) was injected into the HPLC system. The methodologic details for metabolite elution and electrochemical detection were described previously (25, 26).

**Statistical analysis**

Sociodemographic and lifestyle characteristics of case and control subjects were compared with the Fisher exact test for categorical variables or with the Mann-Whitney \( U \) or Wilcoxon rank-sum test for continuous variables. All plasma biomarkers exhibited positively skewed distributions; therefore, to achieve normality, biomarker data were log-transformed (natural log) before analysis. Normality of the transformed data was verified by using the Anderson-Darling test (27). Outlier analysis was performed for each biomarker, stratified by case-control status. Among the case subjects, 4 questionable values that fell beyond 3 SDs from the mean (in both the log-transformed and untransformed data) were considered to be outliers. There were no outliers identified among the control subjects.
Mean log-transformed biomarker concentrations of case and control subjects were compared by using a Student t test, whereas linear regression was used to adjust these comparisons for age, race, educational level, household income, multivitamin supplement intake, number of cigarettes smoked daily, alcohol consumption, caffeine intake, and interval between the end of pregnancy and the blood draw. Crude and adjusted odds ratios and corresponding 95% CIs for the association between plasma biomarkers and case-control status were computed by using logistic regression. The most important predictors were identified by using a “best subset” approach (28). Analyses were performed with use of the SAS statistical package version 8.2 (SAS Institute, Cary, NC).

RESULTS

Study population

Two hundred fifty-six case subjects were contacted to participate in the study; 32 (12.5%) of the case subjects refused to participate. Similarly, one hundred nine control subjects were contacted to participate in the study; 19 (17.4%) of them refused to participate. Two case subjects had pregnancies that resulted in stillbirth; the remaining women had affected pregnancies that ended in a live birth. Selected characteristics of the 224 case subjects and 90 control subjects that participated in this study are presented in Table 1. The sample consisted primarily of white women. At the time of the blood draw, 62.5% of the case subjects were younger than 30 y, and less than one-half reported alcohol consumption (45.1%). No significant difference was observed between the number of case subjects who reported regular multivitamin use (45.1%) and the number of control subjects who reported regular use (46.7%). Smoking was more prevalent among case subjects (28.1%) than control subjects (16.7%; P = 0.043). The mean caffeine intake did not vary significantly between case subjects (44.59 mg/d) and control subjects (44.16 mg/d; P = 0.628). The median time between the end of pregnancy and the blood draw was significantly longer for control subjects (24.5 mo; range 4.8–49.3 mo) than for case subjects (14.9 mo; range 0.1–52.2 mo; P < 0.01). Eighty-nine (98.9%) of the 90 control subjects and 183 (81.7%) of the 224 case subjects participated ≥6 mo after the end of pregnancy (data not shown).

Biomarker analysis

Plasma concentrations for the 7 biomarkers and the SAM-to-SAH ratio are summarized in Table 2. All biomarker concentrations, except for vitamin B-12, were significantly different between case and control subjects. As compared with controls, case subjects had higher mean homocysteine and SAH concentrations and lower mean methionine and SAM concentrations. The mean folic acid concentration for case subjects was significantly lower (10.33 mg/L) than for control subjects (11.99 mg/L). These biomarkers, except for plasma folic acid and adenosine, remained significant at the 5% level after adjusting for age, race, educational level, household income, smoking, multivitamin intake, alcohol consumption, caffeine intake, and time between the end of pregnancy and blood draw. Similar results were obtained when the outliers were included in the analysis.

Multiple linear logistic regression models stratified by multivitamin use were fitted to identify the simultaneous effect of the biomarkers on case or control status (Table 3). All models were adjusted for the mother’s level of education, number of cigarettes smoked daily, and interval between the end of pregnancy and the blood draw. The SAM:SAH was not included in the model because of collinearity with SAM and SAH. When all subjects, regardless of their multivitamin use, were analyzed together, plasma homocysteine, SAH, and, to a lesser extent, methionine were identified as the most important predictive biomarkers by the best-subset selection method. None of the biomarkers were significantly different in multivitamin users than in nonusers (data not shown).

The concentrations of homocysteine, methionine, and SAH were compared between case and control subjects by computing crude odds ratios at various cutoffs on the basis of biomarker percentiles within controls (Table 4). Of the 224 case subjects, 137 (61.2%) had homocysteine concentrations above the 70th percentile of control subjects, and 31.7% had concentrations

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**Table 1**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases (n = 224)</th>
<th>Controls (n = 90)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt;30 y</td>
<td>140 (62.5)</td>
<td>53 (58.9)</td>
<td>0.608</td>
</tr>
<tr>
<td>Race White</td>
<td>176 (78.6)</td>
<td>72 (80.0)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>28 (12.5)</td>
<td>13 (14.4)</td>
<td></td>
</tr>
<tr>
<td>Smoker No</td>
<td>161 (71.9)</td>
<td>75 (83.3)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>63 (28.1)</td>
<td>15 (16.7)</td>
<td>0.043</td>
</tr>
<tr>
<td>Alcohol drinker No</td>
<td>123 (54.9)</td>
<td>44 (48.9)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>101 (45.1)</td>
<td>46 (51.1)</td>
<td>0.382</td>
</tr>
<tr>
<td>Vitamin supplementation No</td>
<td>122 (54.5)</td>
<td>48 (53.3)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>101 (45.1)</td>
<td>42 (46.7)</td>
<td>0.900</td>
</tr>
<tr>
<td>Education Less than high school</td>
<td>32 (14.3)</td>
<td>14 (15.6)</td>
<td></td>
</tr>
<tr>
<td>Completed high school</td>
<td>71 (31.7)</td>
<td>21 (23.3)</td>
<td></td>
</tr>
<tr>
<td>College education Master’s degree and higher</td>
<td>88 (39.3)</td>
<td>42 (46.7)</td>
<td>0.083</td>
</tr>
<tr>
<td>Missing</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Household income $&lt;10 000</td>
<td>30 (13.4)</td>
<td>12 (13.3)</td>
<td></td>
</tr>
<tr>
<td>$10 000–$30 000</td>
<td>81 (36.2)</td>
<td>26 (28.9)</td>
<td></td>
</tr>
<tr>
<td>$30 000–$50 000</td>
<td>50 (22.3)</td>
<td>28 (31.1)</td>
<td></td>
</tr>
<tr>
<td>$&gt;50 000</td>
<td>37 (16.5)</td>
<td>22 (24.4)</td>
<td>0.224</td>
</tr>
</tbody>
</table>

*P value from Fisher’s exact test for categorical variables and Mann-Whitney U or Wilcoxon rank-sum test for continuous variables.

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$1 \pm SD$ (all such values).
above the 95th percentile of control subjects, indicating a shift to the right (higher values) in the plasma homocysteine distribution of case subjects relative to control subjects. The distribution of plasma SAH values in case subjects was also shifted to the right with respect to control subjects: 103 case subjects (46.2%) had plasma SAH concentrations above the 70th percentile of SAH concentrations in control subjects, and 13.5% had concentrations above the 95th percentile of control subjects. Conversely, the distribution of plasma methionine in case subjects was shifted to the left (lower values) with respect to control subjects. Eighty-six cases (38.6%) had methionine concentrations below the 20th percentile of methionine concentrations in control subjects, and 17.9% had concentrations below the 5th percentile of control subjects. These distributional differences remained important after covariate adjustment. Similar results were obtained when the outliers were retained in the analysis.

### DISCUSSION

After controlling for covariates, plasma concentrations of homocysteine and SAH were elevated, and concentrations of SAM, methionine, and SAM:SAH were reduced among case subjects. There were no significant differences in folate, vitamin B-12, or adenosine between case and control subjects. The 95th percentile homocysteine concentration among women aged 20–39 y in the third National Health and Nutrition Examination Survey sample was 10.4 μg/L. In our sample, 34.4% of case subjects and 5.6% of control subjects had greater homocysteine concentrations (29). The factors contributing to this abnormal maternal metabolic profile are unclear, as are the effects on cardiac development.

Possible causes for the altered biomarkers include deficiencies in folate or vitamin B-12 or both, genetic polymorphisms encoding enzymes in the methionine cycle, or exogenous factors. Mean plasma folic acid was not significantly lower among case subjects than control subjects, as would be expected if folate deficiency was strongly contributing. Most pregnancies in this study began after January 1, 1998, when folic acid fortification of grain products was mandated (30). The mean folic acid concentration among our control subjects was 11.99 mg/L (31), which is consistent with the mean postfortification concentrations among women of childbearing age in other studies (32).

The similarity in folic acid concentrations between case and control subjects suggest that case subjects were either more genetically susceptible to having increases in homocysteine and SAH or were more likely to have reduced activity of relevant enzymes. For example, an alteration in the function of, or genes encoding, methionine synthase may reduce the conversion of homocysteine to methionine (Figure 1). Multiple case-control studies have evaluated the association between NTDs and common genetic variants in the methionine synthase gene with inconsistent results (33–36). To our knowledge, the association between methionine synthase activity and congenital heart defects is as yet undetermined. Thus, further studies are needed of genetic variants associated with congenital heart defects that would cause elevated homocysteine.

Our findings of elevated homocysteine among case subjects are consistent with other studies comparing vitamin-dependent homocysteine metabolism among mothers of offspring with orofacial clefts (15) and NTDs (6) with those of mothers with malformed offspring (15). Studies have also found elevated homocysteine concentrations in women who have had preeclampsia (13) and recurrent early miscarriages (14). In those studies, maternal vitamin and homocysteine concentrations were

### TABLE 3

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>OR (95% CI)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine</td>
<td>26.49 (5.42, 129.59)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.27 (0.06, 1.18)</td>
<td>0.0813</td>
</tr>
<tr>
<td>SAH</td>
<td>2.84 (1.19, 6.76)</td>
<td>0.0184</td>
</tr>
<tr>
<td>SAM</td>
<td>0.43 (0.07, 2.72)</td>
<td>0.3712</td>
</tr>
<tr>
<td>Adenosine</td>
<td>1.39 (0.65, 2.98)</td>
<td>0.3964</td>
</tr>
<tr>
<td>Folate</td>
<td>0.89 (0.41, 1.96)</td>
<td>0.7781</td>
</tr>
<tr>
<td>Vitamin B-12</td>
<td>1.35 (0.58, 3.15)</td>
<td>0.4875</td>
</tr>
</tbody>
</table>

1 n = 314. ORs were adjusted for all other biomarkers listed in the table and for education, number of cigarettes smoked per day, and the time interval between the end of pregnancy and the interview. SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

2 P value tests the hypothesis that the odds ratio is equal to one.
measured up to 6 y after the relevant embryonic period (15). Thus, it was not possible to determine whether alterations in biomarkers among nonpregnant women having adverse pregnancy outcomes reflect the biomarker concentrations during organogenesis or early pregnancy loss. However, it is known that temporal changes in homocysteine concentrations are minimal over a 1–2-y time frame (37). Also, we are unable to determine the temporal relation between the alteration in biomarkers and the adverse pregnancy from our findings and those of others. The evidence suggests that these alterations, if not causally related, are associated with an increased risk of having adverse pregnancy outcomes, including congenital heart defects.

Possible mechanisms by which homocysteine may have an embryotoxic effect include oxidative stress and secondary accumulation of SAH, which leads to product inhibition of DNA methyltransferase reactions, DNA hypomethylation, and altered gene expression (38). Lifestyle factors such as alcohol intake and cigarette smoking are associated with increased oxidative stress (39), which can decrease the functional activity of methionine synthase through limiting the availability of reduced vitamin B-12 (46), and homocysteine (47) gradually decrease during pregnancy, at 6 wk after delivery, the concentrations are similar to those before conception or early pregnancy (47). Further, we examined our data to determine whether concentrations of homocysteine and folate correlated with the time interval between the end of pregnancy and the blood draw in case and control subjects. No discernible trend was found. Therefore, it is likely that, on average, the metabolic patterns observed even more than a year after pregnancy reflect stable adult profiles.

We did not examine the relation between cardiac phenotypes and the biomarkers. Particular cardiac phenotypes may be more influenced by altered homocysteine and methionine metabolism by way of different developmental pathways. Another potential source of bias in our study could be the failure to identify cardiac lesions among control pregnancies. Although control subjects were excluded if the medical chart indicated any congenital malformation, echocardiograms were not required to rule out a cardiac lesion.
REFERENCES


Antiulcer drugs promote oral sensitization and hypersensitivity to hazelnut allergens in BALB/c mice and humans

Isabella Schöll, Eva Untersmayr, Noémi Bakos, Franziska Roth-Walter, Andreas Gleiss, George Boltz-Nitulescu, Otto Scheiner, and Erika Jensen-Jarolim

ABSTRACT

BACKGROUND: Hazelnut allergy can be a consequence of sensitization to cross-reactive pollen, especially from the Fagales family. However, severe allergic reactions after ingestion of hazelnuts without associated pollen allergy have been reported. In these cases, oral sensitization by hazelnut ingestion is plausible.

OBJECTIVE: We have reported that antiulcer drugs promote oral sensitization to digestion-labile food allergens. Because hazelnut proteins were sensitive to gastric digestion in our in vitro assay, we aimed to analyze the effect of antiulcer treatment on oral sensitization to hazelnut proteins.

DESIGN: BALB/c mice were fed hazelnut extract with or without antiulcer drugs. In parallel, gastroenterologic patients (n = 153) were screened during antiulcer treatment for specific immunoglobulin (Ig) E to hazelnut and inhalative allergens in vitro and in vivo.

RESULTS: Mice fed hazelnut extract in combination with antiulcer drugs formed anaphylactogenic IgG1 towards hazelnut and developed type 1 skin reactivity to hazelnut extract. In the human study population, 5 of 153 (3.3%) patients developed hazelnut-specific IgE, 4 of 5 developed specific skin reactivity, 3 of 5 had a positive result to oral provocation, and 2 of 5 manifested a food allergy to hazelnut after a 3-mo course of antiulcer treatment. Immunoblot testing with recombinant allergens showed that hazelnut, but not Fagales pollen, was the genuine elicitor in mice and humans.

CONCLUSION: Our experimental and epidemiologic data suggest that the intake of antiulcer drugs may lead to the induction of immediate-type food hypersensitivity toward hazelnut. Am J Clin Nutr 2005;81:154–60.

KEY WORDS Food allergy, hazelnuts, antiulcer drugs, epidemiology, oral sensitization, digestion

INTRODUCTION

The prevalence of food allergy is steadily increasing and now affects 6–8% of children younger than 3 y and ≈2% of the adult population in the industrialized world (1–3). Of the different edibles, hazelnut has been found in several studies to be the most frequent cause of IgE-mediated food allergy, with a prevalence between 21% and 53% in patients with food allergies in Europe (4–7).

Although patients who reported adverse reactions to hazelnuts mainly showed oral symptoms, gastrointestinal and skin reactions were also reported (6, 8). Most important, severe systemic reactions—including shortness of breath, asthma, and hypotension—affect nearly 5% of nut-allergic patients (6, 9). Symptoms have been observed after the consumption of only 6–7 mg hazelnut protein, amounts comparable with those hidden in dietary products (10, 11). Because tree nut allergy is responsible for fatal or near-fatal outcomes, it represents a significant health concern (12).

Two possible mechanisms for the induction of food allergy have been postulated. On the one hand, sensitization can occur via inhalation of airborne allergens. The subsequent symptoms to a food compound are then elicited by cross-reactive immunoglobulin (Ig) E antibodies. On the other hand, food allergens can directly sensitize the organism via the oral route, but it is proposed that a prerequisite for allergenicity of food is its ability to reach the intestinal mucosa in an intact form, which implies survival of the gastric digestion (13).

Hazelnut allergy can be a consequence of primary sensitization with cross-reactive birch pollen allergens. For instance, the major birch pollen allergen Bet v 1 is homologous to Cor a 1 in hazelnut (14). However, severe allergic reactions after ingestion of hazelnuts without any association to tree pollen allergy have been observed (10, 15–17). The role of hazelnut as a potent and independent elicitor of food allergy was further confirmed by double-blind, placebo-controlled food challenges in a multicenter study (8). In these cases, oral sensitization by hazelnut ingestion is plausible. However, it is puzzling that most of the major allergens in hazelnut extracts are not resistant to gastric and pancreatic digestion (18, 19) and, therefore, do not show the features of classic food allergens (20).

For these reasons, we analyzed the effect of antiulcer drugs on oral sensitization to hazelnut proteins. Recently, we reported that antiulcer drugs promote oral sensitization to digestion-labile fish allergens because they prevent peptic degradation of proteins (21). In the present study, we fed our BALB/c mouse food allergy model (21) hazelnut extract in combination with antiulcer medication and examined the resulting immune response in vitro as

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2 Supported by the Austrian Science Fund F018, Project 8. FR–W was supported by Biomay, Vienna.

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well as in vivo. Furthermore, we collected epidemiologic data from gastroenterologic patients who developed hazelnut-specific IgE during antiulcer treatment and identified the genuine allergen responsible for sensitization.

SUBJECTS AND METHODS

Preparation of hazelnut extract

Commercially available raw hazelnuts were used to prepare an extract, as described previously (21). The protein concentration of the extract was determined according to the method of Bradford (22) with the use of a Bio-Rad Protein Assay (Bio-Rad, Munich, Germany).

Simulated gastric fluid (SGF) consisted of 3.2 mg pepsin/mL (Sigma, Steinheim, Germany) in 0.03 mol NaCl/L (adjusted to pH 1.2 with 1 mol HCl/L). Hazelnut extract (50 μL of 3.3 mg/mL) was added to 200 mL SGF at 37 °C and incubated for 0.5, 1, 5, 15, and 30 min. Hazelnut extract in double-distilled water, in SGF at pH 1.2 without pepsin or in SGF at pH 5.0 with pepsin (imitating antacid conditions), was incubated for 2 h. The reaction was quenched with 75 μL of 1 mol NaOH/L and then the tubes were placed on ice. Aliquots of the samples (20 μL) were separated under reducing conditions on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel, and Coomassie brilliant blue staining was performed.

Immunizations of BALB/c mice

Four- to 6-wk-old female BALB/c mice (n = 5 per group) were obtained from the Institute for Laboratory Animal Science and Genetics (University of Vienna) and were treated according to obtained from the Institute for Laboratory Animal Science and Coomassie brilliant blue staining was performed.

Skin tests of immunized mice

On day 217, Evans blue (100 μL of 5 mg/mL) was injected into the tail vein of mice. Subsequently, 30 μL of hazelnut extract (50 μg/mL), hazel pollen allergen Cor a 1 (2.5 μg/mL; Biomay, Vienna), grass pollen allergen Phl p 5 (2.5 μg/mL; Biomay), mast cell degranulation compound 48/80 (20 μg/mL; Sigma), or PBS were administered intradermally into the shaved abdominal skin. After 20 min, mice were killed and skinned. The diameter of the color reaction was measured on the inside of the abdominal skin. The color intensity was determined by using a hand-held reflection densitometer (Vipdens, Brixen, Italy). Skin reactivity indexes were calculated as described previously (25, 26) (diameter of skin reactivity × densitometrical signal intensity).

Passive cutaneous anaphylaxis

Naive mice were shaved on the abdomen, and then 30 μL hazelnut-specific IgG1 (purified from pooled sera from group B mice, see above) (5 μg/mL), standard IgG1 (5 μg/mL), 48/80 histamine release compound (20 μg/mL; Sigma), and 0.9% NaCl as well as glycerol as negative controls were injected intradermally. After 1 h, 100 μL of hazelnut extract (2.5 mg/mL in 0.9% NaCl) with 5 mg/mL Evans blue was injected intravenously, and after another 30 min the mice were killed and skinned. Skin reactivity indexes were calculated as described above.

Patients

Sera of 153 adult patients (mean age: 65.9 y; 66 men and 87 women) from a gastroenterology clinic in Szolnok, Hungary, were collected. None of the patients reported adverse reactions to inhalant or food allergens; however, IgE immunobLOTS were performed to screen their allergic state. Because of dyspepsia or chronic gastritis, patients were treated orally for 3 mo with an H2-receptor blocker—ranitidine (n = 48; Zantac: 2 × 150 mg/d; Pfizer Inc, New York), famotidin (n = 83; Quametul: 40 × 2 mg/d; Medimpex, London), or ranitidine bismuth citrate (n = 5; Pylorid: 2 × 400 mg/d; GlaxoSmithKline, Uxbridge, United Kingdom)—or with the proton pump inhibitor omeprazole (n = 17; Losec: 1 × 20 mg/d; AstraZeneca, Vienna). Patients who received no treatment (n = 50; mean age: 59.9 y) were used as the control group. The study was conducted in accordance with the 2002 guidelines of the Ministry of Health in Hungary for clinical studies, analogous to the Helsinki criteria for clinical studies.

Serum IgE and skin tests in patients

Sera were tested for allergen-specific IgE against hazelnut and inhalative allergens from tree pollen (eg, birch, hazel, and alder),
grass pollen, weed pollen, molds, house dust mites, insect venoms, and animal dander. Immunoblot testing (AllergyScreen; MEDIWISS Analytic, Moers, Germany) was performed before and 3 and 8 mo after the onset of antiulcer treatment. This system allows the detection of IgE by color reactions through formation of precipitates on nitrocellulose test strips. The coloration is directly proportional to the specific antibody content of the serum sample. Evaluation is carried out after complete drying with a CCD camera (Rapid Reader), which assigns the results to the test classes 0–6 of absolute IgE amounts. Results were considered positive at a class of ≥2 (≥0.75 kU/L). Additionally, total IgE was determined before and at the 3-mo time point (ALLERgen Total IgE; Adaltis, Milano, Italy). Patients with hazelnut-specific IgE in their sera were further subjected to skin tests with hazelnut extract (ALK-Abello, Horsholm, Denmark) at the 8-mo time point.

**Oral provocation of patients**

Single-blind, placebo-controlled oral provocation with hazelnut (Test Dose; Lofarma, Milano, Italy) was performed 11–13 mo after the onset of antiacid therapy in the 5 patients with hazelnut-specific IgE. Patients were tested with hazelnut (doses of 0.15, 0.6, 3.0, 15.0, and 60.0 mg/caps) and with talcum (210 mg/caps) as a negative control. The different doses were given at an interval of 60 min. The tests were stopped at the first appearance of symptoms.

**Western blotting and dot blots**

Hazel extract (2 mg/mL) was separated under reducing conditions in a 12% SDS-PAGE and electrobobtled onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). One blot strip was blocked with PBS and 0.5% Tween, and staining was performed with commercially available black ink in PBS and 0.05% Tween (1:1000) to control the blotted protein (27). Remaining blot strips were blocked with buffer (50 mmol/L sodium phosphate buffer, pH 7.5; 0.5% Tween 20, 0.05% bovine serum albumin); incubated with hazelnut-specific mouse IgG concentrate (1:100), mouse preimmune serum (1:100), or human sera (1:10) overnight at 4 °C; washed, and incubated with 125I-labeled sheep-anti mouse Ig (1:1000) or with 125I-labeled anti-human IgE (1:20) overnight. Additionally, mouse IgG was also tested in bitch pollen or mugwort pollen extract (prepared as hazelnut extract). The reaction was visualized by exposing blots to BIOMAX-MS films (Kodak, Rochester, NY). For dot blots, 1 µg/dot of recombinant Bet v 1, Cor a 1, or Art v 1 (Biomay) or 2 µg/dot of hazelnut extract was applied onto nitrocellulose, blocked, and incubated as above with sera from the 5 hazelnut-allergic patients in the antulcer group or from pools of randomly selected bitch pollen–or mugwort-allergic patients.

**Statistical analysis**

IgG1 values are summarized over time for each mouse in each group as the area under the curve and compared between 2 groups by applying a Mann-Whitney U test to these area under the curve values. The 2 comparisons of the sucralfate and the omeprazole group, respectively, to the group fed only with hazelnut were corrected for multiplicity by using the Bonferroni method. P values ≤ 0.05 were considered to be statistically significant. All computations were performed with the use of SPSS software version 11.5 for WINDOWS (SPSS Inc, Chicago).

**RESULTS**

**Digestion assay**

Hazel extract was incubated in SGF with pepst in pH 1.2 for different time intervals (Figure 1). All proteins in our extract were digested within 30 s (lanes 4–8). Incubating the extract in double-distilled water (lane 1), in SGF at pH 1.2 without pepst (lane 2), hazelnut extract in SGF at pH 5.0 with pepst (lane 3), and hazelnut extract incubated at pH 1.2 with pepst for 30 s (lane 4), 1 min (lane 5), 5 min (lane 6), 15 min (lane 7), and 30 min (lane 8). Lane 9 shows the pepst solution without hazelnut.

**Humoral immune response of immunized mice**

Mice were fed with hazelnut extract alone or in combination with the antiulcer drugs sucralfate (an aluminum sucrose hydrogen sulfate) or omeprazole (a proton pump inhibitor). Feedings during treatment with sucralfate or omeprazole induced hazelnut-specific IgG1 in 4 of 5 mice and in 3 of 5 mice, respectively, whereas feedings with hazelnut extract alone did not induce allergen-specific antibodies in any of the mice (Figure 2). A comparison of the induced IgG1 levels (summarized over time as the area under the curve) between the sucralfate group and the group fed hazelnut only is significant (Bonferroni corrected P = 0.032), whereas the data for the omeprazole group showed no significant difference (P = 0.754).

**Purification and characterization of hazelnut-specific IgG antibodies**

Hazel-specific antibodies induced through medication with sucralfate were purified from sera, concentrated, and analyzed in an SDS-PAGE gel. The concentrate mainly contained IgG antibodies, compared with murine IgE and IgG standards (data not shown). The absolute amounts of subclasses present in the eluted fraction were determined by ELISA. The final concentration of IgG1 in the eluate was 5.1 µg/mL, and the content of IgG2a was 1.7 µg/mL. The serum pool before antibody extraction contained 2.5 µg/mL IgG1 and 2.3 µg/mL IgG2a.
Skin tests of mice

The in vivo relevance of the induced IgG1 antibodies was analyzed by type I skin tests in immunized animals. Mice were tested intradermally with hazelnut extract, recombinant Cor a 1 (major hazel pollen allergen), Phl p 5 (major grass pollen allergen), compound 48/80, and PBS. None of the mice immunized with hazelnut extract only (group A) showed a positive skin reaction to hazelnut extract. In contrast, 3 of 5 mice that received hazelnut extract in combination with sucralfate (group B) showed type I skin reactivity to hazelnut. In the group premedicated with omeprazole (group C), only 1 mouse had a positive skin reaction, although 3 of 5 mice had formed hazelnut-specific IgG1 antibodies (Figure 3). No reactivity was achieved with Cor a 1 from hazel pollen or Phl p 5 from grass pollen (Figure 3).

Passive cutaneous anaphylaxis

To examine the biological activity of purified IgG1 induced by feeding hazelnut during sucralfate treatment (group B), these antibodies were used for passive cutaneous anaphylaxis experiments in naive mice (n = 10). Animals were tested intradermally with purified IgG1 or with purchased standard IgG1 with unknown specificity. All mice displayed positive skin reactivity with purified hazelnut-specific IgG1 after intravenous administration of hazelnut extract (Figure 4). In contrast, no reaction was observed when testing with irrelevant IgG1 (data not shown).

Specific IgE of patients treated with antiulcer drugs

A total of 153 gastroenterologic patients with no history of allergy were treated with antiulcer drugs for 3 mo. Sera were examined for hazelnut-specific IgE in immunoblot before and 3 and 8 mo after commencement of antiulcer medication. None of the patients showed hazelnut-specific IgE before the treatment, but 5 patients (3.3%) had developed hazelnut-specific IgE (class of ≥2, >0.75 kU/L) 3 mo after the onset of treatment. These 5 patients showed no cross-reactive sensitizations against early blooming trees, such as birch, alder, or hazel from the Fagales family. None of the patients in the untreated control group (n = 50) developed specific IgE during the observation period. Because of the smaller control group, no statistical significance could be calculated.

Skin testing and oral provocations

In the 5 patients who developed hazelnut-specific IgE, skin-prick tests were performed after 8 mo, and 4 of 5 patients had a positive reaction to hazelnut. Two of these patients reported clinical symptoms after eating hazelnut (oral allergy syndrome) or after consuming chocolate containing hazelnut (acute urticaria) and 3 of them reacted in oral provocation tests with hazelnut (Table 1).

Specificity of murine and human anti-hazelnut antibodies

The binding patterns of purified murine IgG1 of the sucralfate-treated mice and of IgE from human patients, who had developed
hazelnut reactivity during antiulcer treatment, was compared in a hazelnut immunoblot. The antibodies induced through antiulcer medication in mice (panel A, lane 1) predominantly bound to proteins in the molecular mass range of 33 to 65 kDa in hazelnut extract as shown in Figure 5. These proteins are also relevant for IgE binding in the human patients (panel B, lanes 4–8). IgE binding to the higher-molecular-weight hazelnut compounds was also observed after testing in a pool of patients allergic to birch pollen (panel B, lane 10) but not after testing in a mugwort-specific serum pool (panel B, lane 9). To reveal the genuine sensitizer, we performed immunodots using recombinant allergens (panel C). None of the antiulcer medication–treated patients, who developed hazelnut-specific IgE, reacted with the Fagales pollen allergens Betv1 o rC o ra1 (panel C, lanes 4–8), which were strongly recognized by the serum pool of birch pollen–sensitized patients (panel C, lane 10). One of the 5 hazelnut patients reacted with recombinant Art v 1 from mugwort pollen (panel C, lane 7), which was strongly and exclusively recognized by a serum pool of mugwort pollen–sensitized patients (panel C, lane 9).

DISCUSSION

Of the increasing rates of food allergy, the most obvious rise has occurred in peanut and tree nut allergies (28–30). An important difference between these foods is their stability to gastric digestion: some allergens in peanuts have been shown to be resistant to gastric digestion (13, 19). In agreement with the findings of other reports (18, 19), we showed in the present study that hazelnut allergens are degraded within seconds when incubated with SGF. Hazelnut allergy is mainly ascribed to cross-reactivity with birch pollen (16). Nevertheless, several studies have reported severe anaphylactic reactions without an association with pollen allergy (15–17) or even monosensitization to hazelnut (10).

In a recent study we showed that treatment of mice with antiulcer drugs leads to the induction of T helper 2 cell responses against usually harmless dietary proteins (21). By different mechanisms (acid neutralization, proton pump inhibition, or H2-receptor blocking), these medications elevate the gastric pH and prevent peptic degradation of proteins.

**TABLE 1**

<table>
<thead>
<tr>
<th>No.</th>
<th>and sex</th>
<th>Age</th>
<th>0 mo</th>
<th>3 mo</th>
<th>8 mo</th>
<th>0 mo</th>
<th>3 mo</th>
<th>SPT, 8 mo 1</th>
<th>Clinic, 8 mo 4</th>
<th>PROV, 12 mo 5</th>
<th>Medication 6</th>
</tr>
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<tbody>
<tr>
<td>1 M</td>
<td>y</td>
<td>59</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>102</td>
<td>119</td>
<td>3/7</td>
<td>—</td>
<td>—</td>
<td>A</td>
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<tr>
<td>2 M</td>
<td>y</td>
<td>40</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>128</td>
<td>541</td>
<td>6/12</td>
<td>—</td>
<td>3</td>
<td>B</td>
</tr>
<tr>
<td>3 M</td>
<td>y</td>
<td>55</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>210</td>
<td>1000</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>C</td>
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<tr>
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<td>67</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>68</td>
<td>314</td>
<td>6/16</td>
<td>AU</td>
<td>0.6</td>
<td>C</td>
</tr>
<tr>
<td>5 F</td>
<td>y</td>
<td>68</td>
<td>0</td>
<td>5</td>
<td>4</td>
<td>68</td>
<td>314</td>
<td>5/14</td>
<td>OAS</td>
<td>0.6</td>
<td>A</td>
</tr>
</tbody>
</table>

1. HN, hazelnut; IgE, immunoglobulin E; PROV, oral provocation with hazelnut extract; SPT, skin-prick test with hazelnut.
2. Classes of specific IgE: 0 = <0.35 kU/L, 1 = 0.35–0.75 kU/L, 2 = 0.75–3.5 kU/L, 3 = 3.5–17.5 kU/L, 4 = 17.5–50 kU/L, 5 = 50–100 kU/L, and 6 = >100 kU/L.
3. Reaction is given as mm induration/mm erythema.
4. Patient manifested acute urticaria (AU) after eating chocolate containing hazelnut; patient showed oral allergy syndrome (OAS) (local urticaria in mouth) after eating hazelnut.
5. Symptoms appeared within minutes after swallowing the capsule with hazelnut extract in patient 2 (pruritus) and in patients 4 and 5 (urticaria).
6. A, omeprazole; B, ranitidine bismuth citrate; C, ranitidine.

**FIGURE 5.** Specificity of purified mouse immunoglobulin (Ig) G1 and of human IgE from hazelnut-allergic patients treated with antiulcer drugs. A: Hazelnut-specific purified mouse IgG1 was tested on hazelnut extract (lane 1), birch pollen extract (lane 2), and mugwort extract (lane 3). Bound IgG1 was detected by radiolabeled anti-mouse Ig antibody. B: Hazelnut extract blots were incubated with sera from patients (n = 5) who were treated with antiulcer drugs and developed hazelnut-specific IgE (lanes 4–8), with a pool of sera from mugwort pollen–allergic patients (lane 9), with a pool of sera from birch pollen–allergic patients (lane 10), or with buffer (lane 11). C: Dot blots with triplicates of the recombinant allergens Bet v 1, Cor a 1, and Art v 1 or of hazelnut extract (HN) were tested with sera or buffer as in section B. In sections B and C, IgE was detected with radiolabeled anti-human IgE antibody.
Our in vitro digestion experiments showed that, at pH 5.0, peptic digestion of all hazelnut proteins in an extract was hindered. Our working hypothesis was that in this setting intact proteins could reach the mucosa of the intestine, where they are capable of priming sensitization and inducing T helper 2 cell responses. As a proof of concept, we used our BALB/c mouse model and fed hazelnut extract either without or in combination with antiulcer drugs. Sucralfate, which is known to bind and neutralize hydrochloric acid (31), was chosen as an example for an aluminum compound. It might support IgE induction similarly to aluminum hydroxide, which is used routinely as an experimental and clinical adjuvant in vaccine formulations, and induces T helper 2 cell–type responses. The proton pump inhibitor omeprazole was selected as a substance without any adjuvant properties. Similarly to H₂-receptor blockers, omeprazole reduces the net acid output and hinders pepsin activation. Therefore, we considered that both H₂-receptor blockers and proton pump inhibitors should have the same clinical and experimental effects. Most of the mice in both groups treated with antiulcer drugs concomitantly with hazelnut feedings developed hazelnut-specific IgG1 antibodies. The IgG1 levels were more pronounced in the sucralfate-treated group, which may have been due to 1) the timely application in combination with hazelnut extract in the experimental situation, and 2) the possible adjuvant effect of the aluminum compound. The anaphylactogenic capacity of the IgG1 antibodies induced by feedings with sucralfate was shown in passive cutaneous anaphylaxis. It is already known that, at least in the animal model, specific IgG in complex with its antigen can bind directly to FcγRII on mast cells and thereby achieve triggering capacity (32, 33). Although IgE was below the detectable level, type I skin tests were positive in 3 of 5 mice fed hazelnut extract in combination with sucralfate and in 1 of 5 of the omeprazole-treated mice. In these skin tests, Cor a 1 was also included, but the reaction was negative in all mice, probably because the commercially available recombinant Cor a 1 used in our experiments came from hazel pollen and is only 63% homologous to Cor a 1 from hazelnut (34).

To analyze the effect of our observations in a human group, we screened 153 gastroenterologic patients, who were treated for gastric complaints such as dyspepsia or gastritis but were not instructed to follow a special diet. Before antiulcer medication, no hazelnut-specific IgE was present in any of the sera, but 5 patients developed hazelnut-specific IgE during the 3-mo treatment with ranitidine or omeprazole; an untreated control group (n = 50) did not. Thus, the occurrence of hazelnut sensitization in these patients (3.3%) was higher than the prevalence of all tree nut allergies combined in a general population (0.2–0.7% of the US population, 0.38% of the UK population) (29, 35). The levels of specific IgE decreased until the 8-mo investigation, which pointed toward a low memory response. Nevertheless, skin-prick tests at this time point were positive in 4 of the 5 patients. In addition, oral provocation with hazelnut gave positive results in 3 of the 5 patients even at the 12-mo time point after onset of therapy. These reactions may be due to the longer survival time of IgE fixed to mast cells via the high affinity receptor FcεRI compared with IgE in serum (36). Two of these 5 patients reported acute urticaria or oral allergy syndrome after consumption of hazelnuts or chocolate containing hazelnuts. Importantly, none of the 5 patients had or developed de novo IgE specific for cross-reactive airborne allergens from early blooming trees (eg, birch pollen) during our observation period. This suggests that sensitization to hazelnuts in the 5 patients was not due to primary sensitization with cross-reactive pollen. To definitively answer this question, we performed immunodots using recombinant Bet v 1 and Cor a 1 as marker allergens for Fagales pollen sensitization (14, 37) and Art v 1 for mugwort pollen allergy (38). The results indicate that the patients were not pollen sensitized but that hazelnut was the genuine elicitor of IgE formation. Taken together, our data suggest that antiulcer treatment, or other conditions leading to insufficient protein degradation in the stomach, may lead to sensitization with hazelnut proteins without the involvement of an inhalative allergy.

We thank Ing. Magdolina Vermes for excellent technical assistance. IS and EU were responsible for the animal experiments. NB performed the human study. FR-W conducted the digestion experiments. AG carried out the statistical analysis. GB-N and OS helped prepare the manuscript. EJ-J designed the study and edited and reviewed the manuscript. None of the authors had any conflict of interest and no advisory board affiliations with or financial or personal interest in any organization sponsoring the research for this paper.

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Trial of zinc supplements in relation to pregnancy outcomes, hematologic indicators, and T cell counts among HIV-1–infected women in Tanzania

Wafaie W Fawzi, Eduardo Villamor, Gernard I Msamanga, Gretchen Antelman, Said Aboud, Willy Urassa, and David Hunter

ABSTRACT

Background: In observational studies, the zinc status of HIV-infected persons has been associated with both positive and adverse clinical outcomes. Such endpoints may affect the risk of adverse birth outcomes among HIV-infected women.

Objective: We examined the effects of zinc supplements on birth outcomes, hematologic indicators, and counts of T lymphocyte subsets among 400 HIV-infected pregnant women.

Design: Eligible women between 12 and 27 wk of gestation were randomly assigned to daily oral supplementation with either 25 mg Zn or placebo between recruitment and 6 wk after delivery. All women received iron, folic acid, and multivitamin supplements irrespective of the experimental assignment.

Results: We observed no significant differences in birth weight, duration of gestation, or fetal and neonatal mortality between women in the zinc and placebo groups. Hemoglobin concentrations increased between baseline and 6 wk postpartum in both groups. However, the rise in hemoglobin over this period was significantly lower (P = 0.03) in the zinc group (5 ± 17.9 g/L) than in the placebo group (15.2 ± 18.6 g/L). Similarly, the changes in red blood cell count and in packed cell volume over the same period were significantly lower in the zinc group (P < 0.01 and P = 0.01, respectively). Zinc had no effect on CD4+, CD8+, or CD3+ cell counts during the follow-up period.

Conclusion: Because of the lack of beneficial effects of zinc on adverse pregnancy outcomes and the likelihood of negative effects on hemoglobin concentrations, no compelling evidence exists to support the addition of zinc to prenatal supplements intended for pregnant HIV-infected women.

KEY WORDS Zinc, HIV infection, CD4+ cells, CD8+ cells, CD3+ cells, pregnancy, birth weight, preterm infants, small-for-gestational age infants, Tanzania

INTRODUCTION

Poor maternal zinc status is associated with adverse pregnancy outcomes in many observational studies and in early randomized trials with various methodologic limitations (1). In a well-designed placebo-controlled trial among African American women with low plasma zinc concentrations, prenatal zinc supplementation resulted in a significant increase in birth weight and in a longer duration of pregnancy (2). This result suggests that zinc supplementation may be beneficial in developing countries, where pregnant women may be more likely to have suboptimal zinc intakes. In a series of randomized trials in Asia and Latin America, however, supplementation had no beneficial effect on birth weight or the duration of pregnancy (3). It was noted that no trial has been carried out in African settings, where HIV infection is prevalent. Approximately 10–35% of pregnant women in southern Africa are HIV-infected; these women have a higher risk of adverse pregnancy outcomes, including fetal loss (4, 5), low birth weight, preterm birth, and intrauterine growth retardation (6). If zinc supplementation were beneficial for these outcomes, it would constitute a low-cost intervention.

Apart from its potential effects on pregnancy outcomes, the relations between zinc status and HIV-related outcomes are controversial. High zinc intakes were shown to be significantly associated with faster disease progression and higher risks of mortality among men in a prospective cohort study of asymptomatic HIV-infected men in the United States (7, 8). In another US study of HIV-positive men, however, plasma concentrations of zinc were inversely associated with mortality (9). Results from these observational studies are difficult to interpret; confounding of micronutrient status by other variables, such as stage of disease or access to health care, could provide alternative explanations for these observations.

We have shown that multivitamin supplements administered to HIV-infected women during pregnancy significantly decreased the risks of fetal death, preterm delivery, intrauterine growth retardation, and low birth weight and increased hemoglobin and CD4+ cell counts (10). It is important to examine the potential effects of additional inexpensive nutritional interventions, such as zinc supplementation, on these outcomes. We


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enrolled HIV-infected pregnant women in Dar es Salaam, Tanzania, to examine the efficacy of zinc supplements on birth outcomes, including fetal death, birth weight, and duration of pregnancy. We also examined the effect of the supplements on proxy indicators of HIV disease stage, namely hemoglobin concentrations and T lymphocyte subsets.

SUBJECTS AND METHODS

Recruitment and randomization

Pregnant women who were HIV-infected, who resided in Dar es Salaam at the time of the baseline interview, and who intended to stay in the city until delivery and for 1 y thereafter were eligible to enroll in this trial. Eligibility also required an estimated gestational age at randomization of between 12 and 27 wk. HIV-1 serostatus was ascertained from consenting women with the use of 2 enzyme-linked immunoassays (11), and discrepant results were resolved by Western Blot test (Genetic Systems, Redmond, WA). We used a two-stage informed consent procedure. Consent was initially sought for HIV testing as part of prenatal screening at antenatal clinics. HIV-positive women were not asked to enroll in the randomized trial immediately at the time of posttest counseling; instead, we sought their consent to be randomly assigned at a follow-up visit that was scheduled about 3 d later. In this manner, women were allowed time to cope with their positive HIV result and carefully consider participation in the trial, thereby decreasing the probability of dropout after enrollment. Randomization and all subsequent visits took place at a study clinic located at the Muhimbili National Hospital, Dar es Salaam, the main tertiary care hospital in Tanzania. Participants were recruited between September 2000 and October 2002, during which time the prevalence of HIV-1 among antenatal clinic attendees was 11.5%. The study protocol was approved by the Research and Publications Committee at Muhimbili University College of Health Sciences and the Institutional Review Board of the Harvard School of Public Health.

A randomization list was prepared in blocks of 20, and, at enrollment, each eligible woman was assigned to the next numbered bottle of regimen. Women received a daily oral dose of 1 of the 2 interventions from enrollment until the end of the study at 6 wk postpartum: 1) 25 mg Zn as zinc sulfate included in an effervescent citrus-flavored tablet or 2) a similarly constituted zinc-free placebo. All of the experimental tablets were identical in color, taste, and appearance. Women were instructed to dissolve one tablet in water every day. The resulting solutions were colorless and clear; the strong lemon-lime flavor of the solution effectively concealed the metallic taste of zinc and prevented the participants from determining which regimen they were assigned to. The doses used are safe during pregnancy (12). Both active and placebo regimens were prepared by Hermes Arzneimittel GmbH, Munich, Germany.

Standard of care

All women received ferrous sulfate (400 mg, equivalent to 120 mg ferrous Fe) and folate (5 mg) daily and prophylactic chloroquine phosphate (500 mg, equivalent to 300 mg chloroquine base) weekly as per the standard of prenatal care in Tanzania. Given our earlier findings on the benefits of multivitamin supplements among HIV-positive pregnant women (10), all women also received these supplements starting from the time of randomization until the time of delivery, irrespective of zinc treatment assignment. These supplements contained 20 mg thiamine, 20 mg riboflavin, 25 mg vitamin B-6, 100 mg niacin, 50 μg vitamin B-12, 0.8 mg folate, 500 mg vitamin C, and 30 mg vitamin E. As a measure against mother-to-child transmission of HIV, all women were offered a 200-mg dose of nevirapine to be taken at the onset of labor and 2 mg nevirapine/kg to their infant within 72 h of delivery (13).

Study procedures

At the first visit, information on the woman’s age, education level, marital status, socioeconomic conditions, and obstetric history was collected. Also, at baseline and every month thereafter, a study physician conducted a full physical examination, and a research nurse gathered information on health problems during the prior month and obtained anthropometric measurements. Additional clinical assessment and continued counseling and support were provided as needed. The women who had psychosocial problems were referred to a full time research social worker, who had backup support from 2 psychiatrists. The women were asked to bring their regimen bottles at their monthly clinic visits. Nurses counted the remaining pills, and the women’s supply was replenished. Compliance was evaluated on the basis of the number of tablets absent from the returned bottles divided by the total number of tablets the woman should have taken.

Laboratory results were available to the managing physicians, who prescribed treatment if indicated. Using specimens collected at the randomization visit, we carried out routine stool and urine examinations and obtained a complete blood count. Hemoglobin was measured with a CBC Coulter Counter (Coulter Corporation, Miami). The FACScount system (Becton-Dickinson, San Jose, CA) was used to obtain absolute counts of CD3+, CD4+, and CD8+ T lymphocyte subsets. Blood count and lymphocyte subsets analyses were repeated at 6 wk postpartum.

Women were encouraged to deliver at Muhimbili National Hospital, where research midwives were available 24 h a day. A research midwife weighed the infants to the nearest 10 g on a standard beam balance immediately after birth. She also measured birth length on an infant length board, measured head circumference with a nonstretchable tape (both to the nearest 0.1 cm), and weighed placentas after removal of blood clots. Gestational age was based on recall of the date of the last menstrual period at recruitment.

Women who did not come for their monthly appointments were visited at home when possible and were asked to come to the study clinic if their condition allowed. For women who traveled out of Dar es Salaam, we attempted to maintain contact with neighbors and relatives to collect information on the outcome of pregnancy, ie, whether the woman had a live birth or experienced a miscarriage or a stillbirth.

The primary endpoint of the trial was to examine the effect of zinc supplementation on hemoglobin concentrations between enrollment and 6 wk postpartum. A sample size of 150 subjects in each group was calculated to detect a difference in mean hemoglobin concentration based on a two-sample t test for the difference between 2 regimens. We enrolled an additional 100 women for a total of 400 women. An intent-to-treat analysis of treatment effects was used. Of the 400 women who were enrolled in the study, data on birth outcomes were not available for 3
women (2 in the zinc group and 1 in the placebo group) (Figure 1). Of the 397 women with known birth outcomes (miscarriage, stillbirth, or live birth), 366 had live births and were eligible for the analyses of birth weight and prematurity. We had a specific date of delivery for all 366 women; we did not have a birth weight for 8 of the infants because of delivery at home or at another medical facility. There were no differences in the availability of data on gestational age or birth weight between treatment groups. We examined the effects of the supplements on the continuous birth outcomes, namely gestational age and birth weight, length, head circumference, and placental weight. We also examined the effects of the supplements on the risk of miscarriage (defined as delivery before 28 wk of gestation), stillbirth (defined as delivery of a dead baby at or after 28 wk of gestation), and fetal death (all miscarriages and stillbirths). An infant born with any evidence of life, such as breathing or beating of the heart, was considered live born. Perinatal mortality included stillbirths and deaths in the first 7 d of life. Neonatal mortality was defined as deaths among live births in the first 28 d of life. Other categorical outcomes evaluated included a birth weight <2500 g (low birth weight; LBW), a birth weight <2000 g, preterm delivery (<37 wk of gestation), severe preterm delivery (<34 wk of gestation), and small-for-gestational age (SGA), which was defined as a birth weight less than the 10th percentile for gestational age on the basis of the reference of Brenner et al (14).

We conducted the analyses with and without data from twins (n = 10 pairs), and the results were virtually the same. Thus, the results included data from the twins. Twin pregnancies were analyzed as a single outcome; for continuous variables, such as birth weight, the mean of the 2 twin values was used. For categorical variables, such as stillbirth, if either infant had the outcome, the pregnancy was considered to have that outcome. Treatment effects on categorical variables were assessed by calculating relative risks with 95% CIs based on the exact binomial distribution (15). Wilcoxon’s rank-sum test was used for continuous variables (16).

We examined the effect of the vitamin supplements on maternal T cells (absolute counts of CD4+ and CD8+ cells), total white blood cells, total lymphocytes, and hematologic measurements (hemoglobin, red blood cell count, and packed cell volume). We observed no differences between treatment arms in the availability of data on these measurements. For each endpoint, we examined the difference between treatment groups at baseline and at 6 wk postpartum as well as the change between these visits.

We also assessed whether treatment effects on birth outcomes were modified by baseline CD4+ cell counts in 3 strata (<200, 200–499, and ≥500 cells/mm3) and within tertiles of baseline body mass index (BMI; defined as weight at baseline in kilograms divided by the square of height in meters). We used the Breslow-Day test for homogeneity (17) to examine whether treatment effects were significantly different between strata of potential modifiers. All P values reported are two-sided; statistical significance in this study was defined as P < 0.05. All statistical analyses were carried out using the SAS system (version 8.0; SAS Institute, Cary, NC).

RESULTS

We observed no significant differences at baseline between women in the zinc and placebo groups in sociodemographic characteristics (including age, parity, and marital status) or nutritional variables (including weight and midupper arm circumference) (Table 1). Women in the 2 regimen groups were also not significantly different with respect to baseline height, past history of adverse pregnancy outcomes, or malaria. The mean (±SD) duration of follow-up from the time of randomization was 8.6 ± 7.2 mo (median: 6.4 mo). Compliance with the study regimen was high between the time of randomization and delivery (94 ± 10%; median: 99%; interquartile range: 93–100%) and between the time of delivery and the end of follow-up at 6 wk postpartum (80 ± 27%; median: 92%; interquartile range: 69–100%). There were no significant differences in the duration of follow-up or compliance between the 2 experimental groups.

Zinc supplements had no effect on birth weight (P = 0.96); the mean (±SD) weight in this group was 3071 ± 526 g compared with 3085 ± 507 g in the placebo group (Table 2). There were also no significant differences between the 2 groups in birth length (P = 0.87), head circumference (P = 0.27), or duration of gestation (P = 0.99). Zinc had no significant effect on the categorical pregnancy outcomes, including low birth weight (P = 0.87), prematurity <37 wk (P = 0.78), or small-for-gestational age (P = 0.32) (Table 3). There were also no significant effects of the supplements on birth weight <2000 g or gestation age at birth <34 wk (data not shown).

We examined the effect of the supplements on the risks of death during the fetal and early postpartum periods (Table 4). There were 31 observed fetal deaths: 18 in the zinc group and 13
in the placebo group. Compared with the placebo, zinc had no significant effect on fetal loss (relative risk: 1.39; 95% CI: 0.58, 3.86; \( P = 0.36 \)). The supplements also had no significant effects on perinatal (relative risk: 1.36; 95% CI: 0.64, 3.14; \( P = 0.32 \)) or neonatal (relative risk: 1.45; 95% CI: 0.39, 7.22; \( P = 0.46 \)) death.

We next examined the effect of the supplements on T cell counts (Table 6). Zinc had no effect on CD4\(^{+}\), CD8\(^{+}\), or CD3+ cell counts during the follow-up period. As expected during pregnancy, all cell counts increased in both the zinc and placebo groups. At baseline, women in both the zinc and placebo groups had comparable CD4\(^{+}\) cell counts; the rise in cell count was not significantly different in the 2 groups (mean: 95 and 101 cells/mm\(^3\), respectively; \( P = 0.97 \)).

### DISCUSSION

Zinc supplements in combination with other prenatal supplements, including iron, folate, and other vitamins, did not affect the duration of pregnancy or anthropometric indexes of fetal growth in the population of HIV-positive Tanzanian women studied. The supplements had no effect on CD4\(^{+}\) and other T cell counts but resulted in an adverse effect on concentrations of hemoglobin and other hematologic indicators. Our study had several strengths. Given the study’s randomized double-blind design, differences in background characteristics between the 2 experimental groups were reduced to a minimum; hence, confounding by other variables was unlikely. The high compliance with the regimen and the minimal loss to follow-up strengthened the validity of the findings.

Many other factors need to be considered when interpreting the results. There are no published data on plasma zinc status among adults in Tanzania; however, the women in the study were typical of other urban residents in Dar es Salaam with low intakes of animal products and are thus expected to suffer from zinc deficiency given the predominance of staple foods with a low bioavailability of zinc. Using national data on stunting rates and the adequacy of zinc in the national food supply, investigators of the International Zinc Nutrition Consultative Group estimate that 44% of Tanzanians are at risk of inadequate zinc intake (18). It is unlikely that the null effect noted on pregnancy outcomes was due to the concurrent existence of other micronutrient deficiencies given that we provided all women with a multivitamin supplement, including vitamin B complex and vitamins C and E. The latter supplement was provided given beneficial effects observed

---

**TABLE 1**

Baseline characteristics of the study participants according to treatment regimen

<table>
<thead>
<tr>
<th>Baseline characteristic</th>
<th>Zinc group (n = 200)</th>
<th>Placebo group (n = 200)</th>
<th>( P^1 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week of gestation at first visit (wk)</td>
<td>23.0 ± 3.5(^2)</td>
<td>22.5 ± 3.5</td>
<td>0.12</td>
</tr>
<tr>
<td>Age (y)</td>
<td>26.7 ± 4.9</td>
<td>27.0 ± 5.0</td>
<td>0.45</td>
</tr>
<tr>
<td>No. of previous pregnancies</td>
<td>1.6 ± 1.5</td>
<td>1.8 ± 1.8</td>
<td>0.46</td>
</tr>
<tr>
<td>Midupper arm circumference (cm)</td>
<td>26.0 ± 2.9</td>
<td>26.3 ± 3.2</td>
<td>0.38</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>58.9 ± 9.5</td>
<td>60.6 ± 10.6</td>
<td>0.09</td>
</tr>
<tr>
<td>Lacks formal education [% (n)]</td>
<td>6.6 ± 13</td>
<td>6.0 ± 12</td>
<td>0.82</td>
</tr>
<tr>
<td>Has secondary education [% (n)]</td>
<td>19.2 ± 38</td>
<td>16.5 ± 33</td>
<td>0.48</td>
</tr>
<tr>
<td>Is housewife [% (n)]</td>
<td>67.0 ± 132</td>
<td>68.0 ± 136</td>
<td>0.83</td>
</tr>
<tr>
<td>Has male partner [% (n)]</td>
<td>86.4 ± 171</td>
<td>84.0 ± 168</td>
<td>0.51</td>
</tr>
<tr>
<td>Has her own income [% (n)]</td>
<td>33.3 ± 66</td>
<td>32.5 ± 65</td>
<td>0.86</td>
</tr>
<tr>
<td>Has miscarriage [% (n)]</td>
<td>21.9 ± 43</td>
<td>25.1 ± 50</td>
<td>0.46</td>
</tr>
<tr>
<td>Has had stillbirths [% (n)]</td>
<td>4.6 ± 9</td>
<td>4.0 ± 8</td>
<td>0.78</td>
</tr>
<tr>
<td>Primiparous [% (n)]</td>
<td>25.5 ± 50</td>
<td>23.6 ± 47</td>
<td>0.66</td>
</tr>
</tbody>
</table>

\(^1\) Wilcoxon’s rank-sum and chi-square tests for continuous and categorical characteristics, respectively.

\(^2\) \( \bar{x} \) ± SD (all such values).

---

**TABLE 2**

Effect of zinc supplements on continuous outcomes in newborns

<table>
<thead>
<tr>
<th>Outcome(^4)</th>
<th>Zinc group</th>
<th>Placebo group</th>
<th>( P^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of pregnancy (wk)</td>
<td>39.6 ± 2.9 [180]</td>
<td>39.5 ± 3.2 [186]</td>
<td>0.99</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3071 ± 526 [174]</td>
<td>3085 ± 507 [184]</td>
<td>0.96</td>
</tr>
<tr>
<td>Birth length (cm)</td>
<td>48.6 ± 2.5 [153]</td>
<td>48.5 ± 3.1 [162]</td>
<td>0.87</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>34.4 ± 1.5 [154]</td>
<td>34.6 ± 1.4 [162]</td>
<td>0.27</td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td>517 ± 111 [141]</td>
<td>515 ± 115 [147]</td>
<td>0.70</td>
</tr>
</tbody>
</table>

\(^4\) For twin pregnancies, the average value for both twins was used.

\(^2\) Wilcoxon’s rank-sum test.

\(^4\) \( \bar{x} \) ± SD (all such values); \( n \) in brackets.
Neonatal death
Perinatal death
Fetal death

Effect of zinc supplements on fetal loss and early child mortality

Table 4
<table>
<thead>
<tr>
<th>Outcome</th>
<th>Zinc group</th>
<th>Placebo group</th>
<th>Relative risk (95% CI)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preterm &lt; 37 wk</td>
<td>17.2 [31/180]</td>
<td>15.6 [29/186]</td>
<td>1.11 (0.63, 2.04)</td>
<td>0.78</td>
</tr>
<tr>
<td>Low birth weight, &lt; 2500 g</td>
<td>10.9 [19/174]</td>
<td>10.3 [19/184]</td>
<td>1.06 (0.47, 2.38)</td>
<td>0.87</td>
</tr>
<tr>
<td>Small-for-gestational age3</td>
<td>14.4 [25/176]</td>
<td>18.5 [34/184]</td>
<td>0.78 (0.41, 1.38)</td>
<td>0.32</td>
</tr>
</tbody>
</table>

1 For twin pregnancies, the outcome was positive when at least one of the twins had it.
2 Values in brackets represent the number of infants at risk out of the total number of infants.
3 95% CI based on the exact binomial distribution.
4 Fisher’s exact test.

In an earlier trial among HIV-positive women in Dar es Salaam (10). The dose of zinc used was most likely adequate for having an effect, if such an effect existed. We chose a supplementation dose of 25 mg Zn/d because it was only slightly higher than the recommended dietary allowance for adult women in the United States of 15 mg (19, 20) and because studies using doses of zinc between 20 and 30 mg/d have not reported side effects (2, 12). It is possible that doses higher than those used in the above trials may have had a beneficial effect. However, the dose used is already within close range to the tolerable upper intake level for adults (40 mg). Women were enrolled in the study at their first prenatal visit, which was at 22 wk of gestation on average. Although it is possible that a longer duration of supplementation use may have had a significant effect on pregnancy outcomes, the first antenatal visit in Dar es Salaam was relatively earlier than visits in other countries in sub-Saharan Africa (21), and we were interested in enrolling typical pregnant women in Dar es Salaam.

The lack of effect of zinc supplements on birth weight and duration of pregnancy does not agree with the results of a placebo-controlled trial conducted in pregnant African American women who were presumably mostly HIV-negative (2). In the latter group of women, who were apparently healthy but had plasma zinc concentrations below the median at enrollment, daily zinc supplements (25 mg, as used in the Tanzania trial) resulted in greater birth weights and head circumferences. This effect was observed predominantly in women with a BMI < 26. However, similar to our results, 6 of 7 trials in Asia and Latin America showed no effect of zinc supplementation on birth weight; a modest benefit was noted in the Chilean trial. None of the 7 trials indicated an effect on duration of gestation (3).

The effects of zinc (30 mg zinc sulfate) and iron (60 mg as ferrous fumarate) were examined in a recently completed large trial among pregnant women from Nepal; the women were randomly assigned to 1 of 5 groups: 1) folate; 2) folate and iron; 3) folate, iron, and zinc; 4) folate, iron, zinc, and multivitamins; and 5) placebo. All women received daily vitamin A supplements. None of the supplements had a significant effect on fetal loss or perinatal or neonatal deaths (22). Compared with the placebo group, the women who received iron and folate alone had a 14% reduction in LBW and a higher birth weight; however, the women who received zinc in addition to iron and folate had birth weights that were not significantly different from those of the placebo group. The authors attributed this apparent adverse effect of zinc to possible interference with iron absorption by zinc. Although the women who received zinc in our study in Tanzania had a nonsignificantly higher risk of fetal and early postpartum mortality, the study had limited statistical power to examine the efficacy of the supplements on these endpoints.

Concerns about the safety of zinc supplementation in HIV-infected persons were raised on the basis of findings from an observational study among asymptomatic HIV-infected men from the United States. Dietary zinc intake was associated with significantly higher risks of progression to AIDS (7) and mortality (8). In contrast, normalization of plasma zinc concentrations was associated with higher CD4+ cell counts among men who participated in another prospective cohort study (23). Low plasma zinc was also a significant predictor of AIDS mortality in a third study conducted in the United States (9). We found no effect of zinc supplementation on CD4+ or CD8+ cell counts among women in Tanzania. The effects of the supplements on

Table 4
<table>
<thead>
<tr>
<th>Outcome</th>
<th>Zinc group</th>
<th>Placebo group</th>
<th>Relative risk (95% CI)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miscarriage</td>
<td>2.5 [5/198]</td>
<td>1.5 [5/199]</td>
<td>1.68 (0.17, 40.3)</td>
<td>0.50</td>
</tr>
<tr>
<td>Stillbirth7</td>
<td>6.6 [13/198]</td>
<td>5.0 [10/199]</td>
<td>1.31 (0.44, 4.55)</td>
<td>0.53</td>
</tr>
<tr>
<td>Fetal death6</td>
<td>9.1 [18/198]</td>
<td>6.5 [13/199]</td>
<td>1.39 (0.58, 3.86)</td>
<td>0.36</td>
</tr>
<tr>
<td>Perinatal death7</td>
<td>11.6 [23/198]</td>
<td>8.5 [17/199]</td>
<td>1.36 (0.64, 3.14)</td>
<td>0.32</td>
</tr>
<tr>
<td>Neonatal death8</td>
<td>5.6 [10/180]</td>
<td>3.8 [7/186]</td>
<td>1.45 (0.39, 7.22)</td>
<td>0.46</td>
</tr>
</tbody>
</table>

1 For twin pregnancies, the outcome was positive when at least one of the twins had it.
2 Values in brackets represent the number of infants at risk out of the total number of infants.
3 95% CI based on the exact binomial distribution.
4 Fisher’s exact test.
5 Delivery of a dead child at or after 28 wk of gestation.
6 Included stillbirths and miscarriages.
7 Included stillbirths and child deaths between birth and 28 d postpartum for all women with known pregnancy outcomes.
8 Included events between birth and 28 d postpartum among live births.
viral load or clinical outcomes were not examined. We provided iron supplements to all of the women in our trial as per the standard of prenatal care in Tanzania. Iron deficiency anemia, a major public health problem among pregnant women in Tanzania and other developing countries, is a result of low prepregnancy stores, continued inadequate iron intake, malaria and hookworm infections, and increased iron requirements during pregnancy. Prenatal iron supplementation during pregnancy is routine practice given that it has been shown to raise hemoglobin concentrations (24, 25). Although all of the women in our trial, irrespective of experimental group, experienced an increase in hemoglobin and other hematologic indicators, this increase was significantly less among women who received zinc than in those who received placebo. It is possible that zinc supplementation resulted in a negative effect on iron absorption, as previously proposed (26). Women who participated in a randomized trial in Peru who received iron and folic acid alone or iron, folic acid, and zinc had similar hematologic responses (27). In 2 other trials, however, zinc supplementation was apparently associated with a reduced response to iron supplementation. In a trial in Mexico, pregnant women who received multimicronutrient supplements containing iron (60 mg as ferrous sulfate) and zinc (15 mg) experienced a slight decrease in hemoglobin concentrations compared with those who received iron only (28). In the perinatal trial from Nepal mentioned above, increases in hemoglobin concentrations were smaller in the group randomly assigned to receive folic acid plus iron and zinc than in the group who received folic acid and iron only (22). Concerns about a possible adverse effect of zinc supplements on iron absorption were also raised in 2 trials among children in Indonesia, in whom iron and zinc were less efficacious than was iron alone in improving hemoglobin concentrations and iron status (29, 30).

This was a community-based study of women receiving primary health care during pregnancy. The results are generalizable to HIV-infected women attending prenatal care clinics in Tanzania. In light of the lack of beneficial effects of zinc on adverse pregnancy outcomes and the potential for adverse effects of zinc supplementation on hematologic indicators, there is no compelling evidence to add zinc to prenatal supplements intended for pregnant women.

We thank the mothers and children and the field teams—including the nurses, midwives, supervisors, laboratory staff, and administrative staff—who made the study possible. We greatly appreciate the input of the following colleagues: Illuminata Ballonzi, Jenny Coley, Sylvia Kaaya, Roland Kapuka, and Heavengton Mshi. We also acknowledge the valuable encouragement and support of Ines Golly, previously at Hermes Arzneimittel GmbH, Munich, Germany. We thank the authorities at Muhimbili University College of Health Sciences, Muhimbili National Hospital, the City of Dar es Salaam Regional Health Authority, and the Tanzanian National AIDS Control Program for their institutional support.

WWF was the Harvard Principal Investigator of the project and contributed to the study design, study implementation, and data analyses and was primarily responsible for writing the draft of the manuscript. EV contributed to the data management in the field, data analyses, and writing of the paper. GIM contributed to the study design and the day-to-day running of the study in the field. GA supervised the data entry and management in the field. SA and WWF oversaw the laboratory aspects of the study. DH contributed to the study design and provided advice on technical and practical issues during the implementation of the study. All authors contributed to the editing of the final version of the manuscript. None of the sponsors of the study had any role in the study design, data collection, data analysis, data interpretation, or writing of the report.

REFERENCES

### TABLE 5
Effect of zinc supplements on hematologic indicators

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Zinc group</th>
<th>Placebo group</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>100 ± 12 [192]</td>
<td>98 ± 14 [193]</td>
<td>0.07</td>
</tr>
<tr>
<td>6 wk postpartum</td>
<td>112 ± 16 [183]</td>
<td>114 ± 17 [189]</td>
<td>0.29</td>
</tr>
<tr>
<td>Change</td>
<td>11.5 ± 17.9 [175]</td>
<td>15.2 ± 18.6 [182]</td>
<td>0.03</td>
</tr>
<tr>
<td>Red blood cell count</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(× 10^6/mm³)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.55 ± 0.52 [189]</td>
<td>3.48 ± 0.56 [190]</td>
<td>0.14</td>
</tr>
<tr>
<td>6 wk postpartum</td>
<td>4.16 ± 0.09 [182]</td>
<td>4.26 ± 0.65 [187]</td>
<td>0.12</td>
</tr>
<tr>
<td>Change</td>
<td>0.59 ± 0.75 [172]</td>
<td>0.78 ± 0.70 [177]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Packed cell volume (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>30.9 ± 3.4 [189]</td>
<td>30.4 ± 4.0 [190]</td>
<td>0.05</td>
</tr>
<tr>
<td>6 wk postpartum</td>
<td>34.5 ± 4.7 [182]</td>
<td>35.1 ± 4.8 [187]</td>
<td>0.21</td>
</tr>
<tr>
<td>Change</td>
<td>3.4 ± 5.2 [172]</td>
<td>4.6 ± 5.2 [177]</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Wilcoxon’s rank-sum test.

### TABLE 6
Effect of zinc supplements on immune cell counts

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Zinc group</th>
<th>Placebo group</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4⁺ cell count (×10⁶)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>401 ± 203 [140]</td>
<td>415 ± 210 [148]</td>
<td>0.80</td>
</tr>
<tr>
<td>6 wk postpartum</td>
<td>500 ± 251 [142]</td>
<td>525 ± 290 [150]</td>
<td>0.73</td>
</tr>
<tr>
<td>Change</td>
<td>95 ± 126 [106]</td>
<td>101 ± 137 [116]</td>
<td>0.97</td>
</tr>
<tr>
<td>CD8⁺ cell count (×10⁶)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>716 ± 282 [140]</td>
<td>763 ± 347 [148]</td>
<td>0.40</td>
</tr>
<tr>
<td>6 wk postpartum</td>
<td>1004 ± 452 [142]</td>
<td>965 ± 389 [150]</td>
<td>0.71</td>
</tr>
<tr>
<td>Change</td>
<td>279 ± 308 [106]</td>
<td>207 ± 282 [116]</td>
<td>0.11</td>
</tr>
<tr>
<td>CD4⁺.CD8⁺ cell count (×10⁶)</td>
<td>0.63 ± 0.39 [140]</td>
<td>0.64 ± 0.37 [148]</td>
<td>0.65</td>
</tr>
<tr>
<td>6 wk postpartum</td>
<td>0.57 ± 0.37 [142]</td>
<td>0.61 ± 0.36 [150]</td>
<td>0.29</td>
</tr>
<tr>
<td>Change</td>
<td>−0.07 ± 0.19 [106]</td>
<td>−0.05 ± 0.18 [116]</td>
<td>0.23</td>
</tr>
<tr>
<td>CD3⁺ cell count (×10⁶)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1186 ± 382 [140]</td>
<td>1254 ± 456 [148]</td>
<td>0.34</td>
</tr>
<tr>
<td>6 wk postpartum</td>
<td>1610 ± 630 [142]</td>
<td>1588 ± 557 [150]</td>
<td>0.89</td>
</tr>
<tr>
<td>Change</td>
<td>410 ± 420 [106]</td>
<td>330 ± 400 [116]</td>
<td>0.25</td>
</tr>
<tr>
<td>Total lymphocyte count (×10⁶)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1731 ± 539 [189]</td>
<td>1831 ± 656 [190]</td>
<td>0.17</td>
</tr>
<tr>
<td>6 wk postpartum</td>
<td>1924 ± 686 [182]</td>
<td>1895 ± 607 [187]</td>
<td>0.79</td>
</tr>
<tr>
<td>Change</td>
<td>168 ± 793 [172]</td>
<td>48 ± 842 [177]</td>
<td>0.79</td>
</tr>
<tr>
<td>White blood cell count (×10⁶)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>5166 ± 1619 [192]</td>
<td>5329 ± 1814 [193]</td>
<td>0.38</td>
</tr>
<tr>
<td>6 wk postpartum</td>
<td>4972 ± 1968 [183]</td>
<td>4951 ± 1540 [189]</td>
<td>0.74</td>
</tr>
<tr>
<td>Change</td>
<td>−322 ± 2252 [175]</td>
<td>−413 ± 2196 [182]</td>
<td>0.86</td>
</tr>
</tbody>
</table>

* Wilcoxon’s rank-sum test.

*1 ± SD (all such values); n in brackets.
Sustained effect of short-term calcium supplementation on bone mass in adolescent girls with low calcium intake

Roni P Dodiuk-Gad, Geila S Rozen, Gad Rennert, Hedy S Rennert, and Sophia Ish-Shalom

ABSTRACT
Background: The effect of short-term calcium supplementation on peak bone mass in adolescent girls is not completely defined. In our previous double-blind, placebo-controlled, calcium-supplementation study (1000 mg calcium carbonate/d), we showed that calcium supplementation of postmenarcheal girls with low calcium intakes enhances bone mineral acquisition.

Objective: The objective of this follow-up study, conducted 3.5 y after the end of calcium supplementation, was to investigate the sustained effect of calcium supplementation on bone mineral mass.

Design: Anthropometric data, nutrient intakes, and bone variables were reassessed in 96 of the 100 adolescent girls whose data had been studied at the end of the supplementation period. Bone mineral content and bone mineral density (BMD) of the total body, lumbar spine, and femoral neck were determined by dual-energy X-ray absorptiometry.

Results: The calcium-supplemented group tended to have a greater accretion of total-body BMD (TBBMD) than did the control group 3.5 y after the end of supplementation. The finding was statistically significant in the active-treatment cohort (n = 17 in the calcium-supplemented group and 28 in the placebo group), who had a compliance rate of ≥75% during the intervention study. In a multivariate linear-regression analysis, TBBMD accretion from the beginning of the intervention study to the follow-up study in the active-treatment cohort was attributed to calcium supplementation and to the time since inclusion in the initial study.

Conclusion: Calcium supplementation for 1 y in postmenarcheal girls with low calcium intakes may provide a sustained effect on the basis of TBBMD measurements in participants with compliance rates of ≥75%.

KEY WORDS Low calcium intake, calcium supplementation, bone density, adolescents, postmenarcheal girls, follow-up study

INTRODUCTION
Osteoporotic fractures are a common cause of morbidity in the Western world (1–4). At present there are 2 important approaches to the prevention of this disease: increasing bone mass acquisition at skeletal maturity and reducing the rate of bone loss after menopause (5–14). A maximal peak bone mass at skeletal maturity is considered the best protection against age-related bone loss and subsequent fracture risk (1). Calcium intake seems to be an important determinant of peak bone mass. Recent calcium-supplementation trials in children and adolescents have confirmed a positive effect of calcium intake on bone mineral accretion (15–23). However, an increase in bone mineral density (BMD) in childhood will not prevent osteoporosis in later life unless it is sustained. Several follow-up studies were conducted to confirm whether the positive effect of calcium supplementation on bone mass during growth would be sustained after the treatment is withdrawn and, therefore, be translated into a decreased risk of osteoporosis in later life.

No sustained effect was observed in some previous follow-up studies (24–26). This led to an assumption that the effect of calcium supplementation on bone mineral acquisition reflected a transient reduction in bone remodeling and that there was little benefit from calcium supplementation on bone mineral acquisition. If this assumption proves to be correct, a short-term increase in calcium intake in children would provide no protection against osteoporosis. However, in the study by Bonjour et al (27), a statistically significant increase in the mean BMD of the 6 skeletal sites between calcium-supplemented and control subjects was maintained 3.5 y after the end of supplementation. A significant difference in favor of the supplemented group was also seen with respect to mean BMC and mean bone area. The participants of that study were prepubertal girls with a mean age of 8 y.

Unlike the results of previous studies, the results of that study suggested that calcium supplementation may increase bone mass not only by inhibiting the process of remodeling but also by stimulating bone modeling. In a former study by our group, the effect of calcium supplementation on bone mass accretion during adolescence was evaluated in a double-blind, placebo-controlled, calcium-supplementation study (1000 mg calcium carbonate/d) (28). One hundred postmenarcheal girls with low calcium intakes (<800 mg/d) completed a 1-y intervention trial. At the end of the study, the supplemented group displayed a higher accretion of total-body BMD (TBBMD) than did the control group (3.8 ± 0.3% compared with 3.10 ± 0.29% in the placebo group; P = 0.04).

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The purpose of the present study was to evaluate the long-term effect of the calcium-supplementation trial on bone mass by investigating whether the difference in bone accumulation between the groups persisted 3.5 y after the withdrawal of the calcium supplement.

**SUBJECTS AND METHODS**

In the present study, there were 2 study periods: an intervention study (study A: a 1-y double-blind, placebo-controlled, calcium-supplementation study; 28) and a follow-up study conducted 3.5 ± 0.7 y after the termination of the intervention study (study B). The characteristics of the participants and the methods used in study A are detailed in the previous publication (28). The important aspects are described below.

**Study sample**

One hundred girls (49 calcium-supplemented and 51 placebo) completed study A. Ninety-seven girls who had successfully completed the trial agreed to take part in the follow-up study, which was conducted 3.5 ± 0.7 y after the termination of the intervention study. One of the girls was excluded from the follow-up study because she was pregnant during the period between the 2 studies. The final cohort in study B consisted of 96 participants: 49 girls in the original calcium-supplemented group and 47 girls in the placebo group. The Hospital Review Board approved the study protocol, and informed consent was obtained from the participants.

During the intervention study, the calcium-supplemented group received 1000 mg elemental Ca/d in the form of chewable calcium carbonate tablets (Tevasidan; Teva Pharmaceuticals Industry, Petah-Tiqva, Israel). The control group received identically shaped placebo tablets provided by the same manufacturer. Calcium supplement use was assessed monthly by trained dietitians.

**Assessment of dietary intake**

Individual dietary intake was assessed with a semiquantitative food-frequency questionnaire, which included all known commercial dairy products and all typical foods eaten by teenagers: fast food, snacks, etc. Nutritional analysis was performed by using a database created from Israeli food-composition tables from the Ministry of Health, information from local food manufacturers, and foreign tables of food composition. The same food-frequency questionnaire was used in study A and study B.

**Assessment of influencing factors**

Lifestyle habits and medical status were assessed with a questionnaire. The aim was to evaluate the different possible influencing factors on bone mass during the period between the 2 studies, such as bone fractures, medication use, smoking, and physical activity.

**Weight and height**

The weight of the girls was measured while they were wearing minimal clothing and no shoes. The weight was determined to the nearest 0.10 kg. Standing height was recorded to the nearest 0.10 cm. Weight and height were evaluated with the use of the same equipment and by the same operator throughout both study periods. Weight and height measurements were performed at baseline, 6 mo, and 12 mo in study A and 3.5 y after the intervention study was discontinued in study B.

**Evaluation of bone status**

Bone mineral mass values determined at the beginning of the calcium-supplementation trial were used as baseline values in the current follow-up study. Bone mineral content (BMC; in g) and BMD (in g/cm²) as estimates of bone mass were determined by dual-energy X-ray absorptiometry (Lunar DPX scanner; Lunar Corp, Madison, WI). Bone mineral measurements included a total body scan and 2 skeletal sites: lumbar spine (L2-L4) and femoral neck. The precision error in vivo was ±0.6%, 0.9%, and 1.5%, respectively, for the spine scans (L2-L4) at slow, medium, and fast speeds, whereas the error was 1.2% and 1.5–2%, respectively, for the femur scans at slow and medium speeds. The precision of total-body BMD was 0.5% in vitro and in vivo (29, 30). The CV of the BMD measurement at these sites (as determined in healthy young adults) is between 1% and 1.6%. The scans were acquired by using the appropriate scan mode for the patient’s weight. Bone mineral measurements were performed with the same machine and by the same operator throughout both study periods. Densitometric evaluations were performed in study A at baseline, 6 mo, and 12 mo, and in study B 3.5 y after the intervention was discontinued.

**Statistical analysis**

Comparisons of percentage increases and absolute increases in bone measurements between the calcium-supplemented and the control groups were tested by using the Mann-Whitney U test because of the nonnormal distribution of the data. When the baseline data were controlled for, a regression analysis was performed by using the ranks of the dependent variables. For other variables, a two-tailed Student’s t test was used for a comparison of means. A multivariate analysis using linear regression was used to test the potential effects of different variables on the prediction of percentage gains in BMD and BMC in the different skeletal areas. The differences in the anthropometric and osteodensitometric variables were analyzed both in terms of the intention-to-treat cohort (accounting for all subjects who entered the follow-up study) and an active-treatment cohort (which included the subjects who participated in study B and consumed ≥75% of the prescribed supplement during study A). The intention-to-treat cohort consisted of 96 participants: 49 in the calcium-supplemented group and 47 in the placebo group. The active-treatment cohort consisted of 45 participants: 17 in the calcium-supplemented group and 28 in the placebo group. All results are given as means ± SEMs. The level of significance for all tests was P ≤ 0.05. Statistical analysis was performed by using SPSS/PC, version 11.5 (SPSS Inc, Chicago).

**RESULTS**

**Characteristics of the group at inclusion in the intervention study**

The characteristics of the study and control groups at inclusion in the intervention study (study A) are shown in Table 1 and Table 2. At inclusion in study A, there were no significant differences between the study and control groups for age, body
Compliance during the intervention study

One hundred twelve participants entered the calcium-supplementation trial (study A); only 100 subjects (49 calcium-supplemented and 51 placebo) completed the trial. Ninety-six girls who had successfully completed the trial took part in the follow-up study (49 calcium-supplemented and 47 placebo). During the calcium-supplementation trial, compliance was evaluated monthly by pill count. The mean compliance rate of the entire cohort was 67.33 ± 2.5% during study A. There was no significant difference between the compliance rate of the calcium-supplemented group and the control group.

Effect of calcium supplementation on bone mass

The aim of this research was to determine the net gain in bone mass accumulation since the time of inclusion in the calcium-supplementation study. Consequently, the term percentage accretion in this article refers to the percentage accretion in BMD or BMC since the beginning of the intervention study (study A) (28). The analyses were conducted for 2 different cohorts: the intention-to-treat cohort, which included all of the 96 participants, and the active-treatment cohort, which included the subjects who participated in the follow-up study and consumed ≥75% of the prescribed supplement during the intervention study.

Bone mineral density: percentage accretion

At the end of study A, the calcium-supplemented girls had a higher accretion of TBBMD than did the control group (3.8 ± 0.3% compared with 3.1 ± 0.29%; P = 0.04) (Table 3). In the follow-up study (study B), TBBMD accretion tended to be higher in the calcium-supplemented group; however, statistical significance was shown only for the active-treatment cohort, who had

### TABLE 1
Characteristics of the 96 girls at baseline, at the end of 1 y of calcium supplementation, and 3.5 y after discontinuation of the intervention

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo group (n = 47)</th>
<th>Calcium group (n = 49)</th>
<th>Placebo group (n = 28)</th>
<th>Calcium group (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (y)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>14.85 ± 0.11</td>
<td>14.86 ± 0.09</td>
<td>15.07 ± 0.15</td>
<td>15.10 ± 0.14</td>
</tr>
<tr>
<td>1 y</td>
<td>15.85 ± 0.11</td>
<td>15.86 ± 0.09</td>
<td>16.07 ± 0.15</td>
<td>16.10 ± 0.14</td>
</tr>
<tr>
<td>3.5 y</td>
<td>19.29 ± 0.17</td>
<td>19.67 ± 0.14</td>
<td>19.51 ± 0.22</td>
<td>19.79 ± 0.21</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>20.54 ± 0.47</td>
<td>20.75 ± 0.41</td>
<td>19.84 ± 0.44</td>
<td>20.17 ± 0.67</td>
</tr>
<tr>
<td>1 y</td>
<td>20.96 ± 0.50</td>
<td>21.20 ± 0.43</td>
<td>20.04 ± 0.44</td>
<td>21.17 ± 0.83</td>
</tr>
<tr>
<td>3.5 y</td>
<td>21.61 ± 0.58</td>
<td>21.48 ± 0.38</td>
<td>21.16 ± 0.58</td>
<td>22.04 ± 0.76</td>
</tr>
<tr>
<td><strong>Height (cm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>160.79 ± 0.77</td>
<td>161.26 ± 0.94</td>
<td>160.45 ± 0.96</td>
<td>162.04 ± 1.82</td>
</tr>
<tr>
<td>1 y</td>
<td>162.04 ± 0.80</td>
<td>162.03 ± 0.91</td>
<td>161.53 ± 0.98</td>
<td>162.72 ± 1.74</td>
</tr>
<tr>
<td>3.5 y</td>
<td>162.61 ± 0.82</td>
<td>162.99 ± 0.94</td>
<td>162.04 ± 0.96</td>
<td>163.65 ± 1.81</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>53.21 ± 1.40</td>
<td>54.04 ± 1.23</td>
<td>51.11 ± 1.26</td>
<td>53.09 ± 2.09</td>
</tr>
<tr>
<td>1 y</td>
<td>54.78 ± 1.54</td>
<td>55.12 ± 1.30</td>
<td>52.38 ± 1.36</td>
<td>55.00 ± 2.17</td>
</tr>
<tr>
<td>3.5 y</td>
<td>57.34 ± 1.78</td>
<td>57.12 ± 1.18</td>
<td>55.64 ± 1.70</td>
<td>59.24 ± 2.52</td>
</tr>
<tr>
<td><strong>Time since menarche (y)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.16 ± 0.12</td>
<td>2.19 ± 0.14</td>
<td>2.24 ± 0.19</td>
<td>2.47 ± 0.24</td>
</tr>
<tr>
<td>1 y</td>
<td>3.16 ± 0.12</td>
<td>3.19 ± 0.14</td>
<td>3.24 ± 0.19</td>
<td>3.47 ± 0.24</td>
</tr>
<tr>
<td>3.5 y</td>
<td>6.56 ± 0.17</td>
<td>6.83 ± 0.17</td>
<td>6.69 ± 0.25</td>
<td>7.15 ± 0.21</td>
</tr>
<tr>
<td><strong>Calcium intake (mg/d)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>586.70 ± 22.50</td>
<td>580.22 ± 24.49</td>
<td>590.36 ± 28.52</td>
<td>611.18 ± 40.20</td>
</tr>
<tr>
<td>3.5 y</td>
<td>720.63 ± 52.45</td>
<td>599.68 ± 36.83</td>
<td>712.20 ± 76.94</td>
<td>620.30 ± 64.09</td>
</tr>
<tr>
<td><strong>Energy intake (kcal)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1747.81 ± 84.64</td>
<td>1836.21 ± 98.84</td>
<td>1725.71 ± 99.12</td>
<td>1846.29 ± 169.92</td>
</tr>
<tr>
<td>3.5 y</td>
<td>1763.67 ± 109.32</td>
<td>1499.55 ± 87.70</td>
<td>1714.04 ± 120.84</td>
<td>1427.62 ± 134.97</td>
</tr>
</tbody>
</table>

1 All values are ± SEM. The analyses were conducted in 2 different cohorts: the intention-to-treat cohort, which included all of the 96 participants, and the active-treatment cohort, which included the subjects who participated in the follow-up study and consumed ≥75% of the prescribed supplement during the intervention study.

2 Significantly different from the placebo group, P = 0.036 (Student’s t test).

mass index (BMI; in kg/m²), height, body weight, time since menarche, calcium intake, energy intake, and bone mineral measures of the lumbar spine (L2-L4), femoral neck, and total body.

Characteristics of the group at the follow-up study

The characteristics of the study and control groups at the follow-up study (study B) are shown in Tables 1 and 2. At the inclusion in study B, there were no significant differences between the study and control groups for age, BMI, height, body weight, time since menarche, calcium intake, and bone mineral measures of the lumbar spine (L2-L4), femoral neck, and total body. Only energy intake was significantly different between groups, with the placebo group having a higher energy intake than the calcium-supplemented group.

Effect of calcium supplementation on bone mass

The aim of this research was to determine the net gain in bone mass accumulation since the time of inclusion in the calcium-supplementation study. Consequently, the term percentage accretion in this article refers to the percentage accretion in BMD or BMC since the beginning of the intervention study (study A) (28). The analyses were conducted for 2 different cohorts: the intention-to-treat cohort, which included all of the 96 participants in the follow-up study, and the active-treatment cohort, which included the subjects who participated in the follow-up study and consumed ≥75% of the prescribed supplement during the intervention study.

Bone mineral density: percentage accretion

At the end of study A, the calcium-supplemented girls had a higher accretion of TBBMD than did the control group (3.8 ± 0.3% compared with 3.1 ± 0.29%; P = 0.04) (Table 3). In the follow-up study (study B), TBBMD accretion tended to be higher in the calcium-supplemented group; however, statistical significance was shown only for the active-treatment cohort, who had
Total-body BMD (g/cm²)

<table>
<thead>
<tr>
<th></th>
<th>Placebo group (n = 47)</th>
<th>Calcium group (n = 49)</th>
<th>Placebo group (n = 28)</th>
<th>Calcium group (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>1.05 ± 0.01</td>
<td>1.04 ± 0.01</td>
<td>1.05 ± 0.01</td>
<td>1.05 ± 0.02</td>
</tr>
<tr>
<td>1 y</td>
<td>1.09 ± 0.01</td>
<td>1.08 ± 0.01</td>
<td>1.09 ± 0.01</td>
<td>1.09 ± 0.02</td>
</tr>
<tr>
<td>3.5 y</td>
<td>1.11 ± 0.01</td>
<td>1.11 ± 0.01</td>
<td>1.11 ± 0.01</td>
<td>1.12 ± 0.18</td>
</tr>
</tbody>
</table>

Lumbar spine (L2-L4) BMD (g/cm²)

<table>
<thead>
<tr>
<th></th>
<th>Placebo group (n = 47)</th>
<th>Calcium group (n = 49)</th>
<th>Placebo group (n = 28)</th>
<th>Calcium group (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>1.09 ± 0.19</td>
<td>1.08 ± 0.02</td>
<td>1.07 ± 0.03</td>
<td>1.10 ± 0.03</td>
</tr>
<tr>
<td>1 y</td>
<td>1.12 ± 0.02</td>
<td>1.12 ± 0.02</td>
<td>1.10 ± 0.02</td>
<td>1.13 ± 0.03</td>
</tr>
<tr>
<td>3.5 y</td>
<td>1.15 ± 0.02</td>
<td>1.15 ± 0.02</td>
<td>1.14 ± 0.03</td>
<td>1.17 ± 0.03</td>
</tr>
</tbody>
</table>

Femoral neck BMD (g/cm²)

<table>
<thead>
<tr>
<th></th>
<th>Placebo group (n = 47)</th>
<th>Calcium group (n = 49)</th>
<th>Placebo group (n = 28)</th>
<th>Calcium group (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.99 ± 0.17</td>
<td>0.99 ± 0.01</td>
<td>0.99 ± 0.02</td>
<td>1.00 ± 0.03</td>
</tr>
<tr>
<td>1 y</td>
<td>1.00 ± 0.02</td>
<td>1.01 ± 0.01</td>
<td>1.00 ± 0.03</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td>3.5 y</td>
<td>0.99 ± 0.02</td>
<td>1.01 ± 0.02</td>
<td>1.00 ± 0.02</td>
<td>1.01 ± 0.03</td>
</tr>
</tbody>
</table>

Total-body BMC (g)

<table>
<thead>
<tr>
<th></th>
<th>Placebo group (n = 47)</th>
<th>Calcium group (n = 49)</th>
<th>Placebo group (n = 28)</th>
<th>Calcium group (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>819.00 ± 17.70</td>
<td>820.53 ± 18.48</td>
<td>808.25 ± 22.66</td>
<td>841.06 ± 35.66</td>
</tr>
<tr>
<td>1 y</td>
<td>860.34 ± 20.23</td>
<td>860.29 ± 19.17</td>
<td>841.86 ± 24.88</td>
<td>883.88 ± 38.19</td>
</tr>
<tr>
<td>3.5 y</td>
<td>896.13 ± 20.55</td>
<td>895.74 ± 18.94</td>
<td>884.18 ± 25.67</td>
<td>927.12 ± 39.79</td>
</tr>
</tbody>
</table>

Lumbar spine (L2-L4) BMC (g)

<table>
<thead>
<tr>
<th></th>
<th>Placebo group (n = 47)</th>
<th>Calcium group (n = 49)</th>
<th>Placebo group (n = 28)</th>
<th>Calcium group (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>41.82 ± 1.04</td>
<td>40.69 ± 1.11</td>
<td>41.05 ± 1.27</td>
<td>41.86 ± 1.98</td>
</tr>
<tr>
<td>1 y</td>
<td>43.32 ± 1.03</td>
<td>42.70 ± 1.14</td>
<td>42.55 ± 1.26</td>
<td>43.84 ± 2.24</td>
</tr>
<tr>
<td>3.5 y</td>
<td>45.82 ± 1.15</td>
<td>45.25 ± 1.12</td>
<td>45.03 ± 1.45</td>
<td>46.39 ± 2.52</td>
</tr>
</tbody>
</table>

Femoral neck BMC (g)

<table>
<thead>
<tr>
<th></th>
<th>Placebo group (n = 47)</th>
<th>Calcium group (n = 49)</th>
<th>Placebo group (n = 28)</th>
<th>Calcium group (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>4.40 ± 0.94</td>
<td>4.34 ± 0.09</td>
<td>4.3 ± 0.11</td>
<td>4.42 ± 0.19</td>
</tr>
<tr>
<td>1 y</td>
<td>4.54 ± 0.10</td>
<td>4.54 ± 0.08</td>
<td>4.46 ± 0.12</td>
<td>4.66 ± 0.16</td>
</tr>
<tr>
<td>3.5 y</td>
<td>4.55 ± 0.10</td>
<td>4.54 ± 0.09</td>
<td>4.46 ± 0.12</td>
<td>4.64 ± 0.18</td>
</tr>
</tbody>
</table>

TABLE 3

Percentage gain in bone mineral density (BMD) from baseline to the end of each of the 2 studies

<table>
<thead>
<tr>
<th></th>
<th>Intention-to-treat cohort</th>
<th>Active-treatment cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo group (n = 47)</td>
<td>Calcium group (n = 49)</td>
</tr>
<tr>
<td>Study A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total body</td>
<td>3.10 ± 0.29</td>
<td>3.80 ± 0.30</td>
</tr>
<tr>
<td>Femoral neck</td>
<td>1.52 ± 0.44</td>
<td>2.00 ± 0.51</td>
</tr>
<tr>
<td>Lumbar spine</td>
<td>2.93 ± 0.45</td>
<td>3.66 ± 0.35</td>
</tr>
<tr>
<td>Study B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total body</td>
<td>5.82 ± 0.52</td>
<td>6.48 ± 0.46</td>
</tr>
<tr>
<td>Femoral neck</td>
<td>0.40 ± 0.88</td>
<td>1.08 ± 0.55</td>
</tr>
<tr>
<td>Lumbar spine</td>
<td>6.02 ± 0.71</td>
<td>6.52 ± 0.50</td>
</tr>
</tbody>
</table>

The analyses were conducted in 2 different cohorts: the intention-to-treat cohort, which included all of the 96 participants, and the active-treatment cohort, which included the subjects who participated in the follow-up study and consumed ≥75% of the prescribed supplement during the intervention study. The term percentage gain refers to the percentage accretion in bone mass since the time of inclusion in the calcium-supplementation study (study A).
in the analysis (Table 3). The percentage accretion of BMD in the femoral neck tended to be higher in the calcium-supplemented group than in the placebo group in both the initial and current studies, but the differences between the groups were not statistically significant, even when the active-treatment cohort was included in the analysis (Table 3).

**Bone mineral content: percentage accretion**

The accretion of BMC in the femoral neck and lumbar spine tended to be higher in the calcium-supplemented group than in the placebo group in both initial and current studies, but the differences between the groups were not statistically significant, even in analyzing the active-treatment cohort. Total-body BMC accretion tended to be higher in the placebo group than in the calcium-supplemented group in both initial and current studies; this trend was reversed in analyzing the active-treatment cohort (Table 4).

**Regression model**

A multivariate analysis using linear regression was conducted to determine the most important parameters affecting accretion of TBBMD in the active-treatment cohort from the beginning of study A to study B. The model included parameters from the period between the studies, such as physical activity, calcium intake, smoking, use of contraceptives, weight change, and height change, as well as baseline data at study A: age, time since menarche, TBBMD, attribution to a group, height, and weight. In the final regression model, attribution to the calcium-supplemented group and time since inclusion in the initial study were statistically significant positive predictors of TBBMD accretion, whereas the age and weight at inclusion in the initial study were statistically significant negative predictors (Table 5).

**Influencing factors**

In determining the possible different effects on bone density during the period between the initial study and the follow-up study, no significant differences between the study and the control groups were found in the active-treatment cohort for weight change, BMI change, smoking, use of contraceptives and other medications, physical activity, and time since the initial study (data not shown). The same results were shown when the different effects on bone density in the intention-to-treat cohort were analyzed.

**DISCUSSION**

In recent years, several calcium-supplementation trials in children and adolescents were conducted to evaluate whether a positive effect on bone mass is sustained after discontinuation of calcium supplementation, which is ultimately translated into higher peak bone mass (24–27). Most clinical trials were unable to detect any differences in bone mass between treatment and control groups after supplementation ceased (24–26). This led to the assumption that the effect of calcium supplementation on bone mineral gain appears to reflect a transient reduction in bone turnover rate. If this observation is confirmed, it will minimize the importance of consistency in maintaining an adequate calcium intake to decrease the risk of fractures in later life. Unlike those previous studies, Bonjour et al (27) reported a significant difference in mean BMD in 6 skeletal sites between the treatment and control groups, which remained for 3.5 y after the end of the supplementation. Moreover, a significant difference in favor of the supplemented group was also seen with respect to mean BMC and mean bone area. The participants of that study were prepubertal girls with a mean age of 8 y.

---

**Table 4**

Percentage gain in bone mineral content (BMC) from baseline to the end of each of the 2 studies

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Placebo group</th>
<th>Calcium group</th>
<th>P</th>
<th>P&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intention-to-treat cohort</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total body</td>
<td>4.76 ± 0.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.63 ± 0.42</td>
<td>0.31</td>
<td>0.31</td>
</tr>
<tr>
<td>Femoral neck</td>
<td>3.33 ± 0.84</td>
<td>4.30 ± 0.86</td>
<td>0.15</td>
<td>0.16</td>
</tr>
<tr>
<td>Lumbar spine (L2–L4)</td>
<td>3.87 ± 0.62</td>
<td>4.52 ± 0.48</td>
<td>0.09</td>
<td>0.11</td>
</tr>
<tr>
<td>Active-treatment cohort</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total body</td>
<td>4.10 ± 0.65</td>
<td>5.11 ± 0.78</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>Femoral neck</td>
<td>3.54 ± 1.30</td>
<td>6.25 ± 1.92</td>
<td>0.09</td>
<td>0.07</td>
</tr>
<tr>
<td>Lumbar spine (L2–L4)</td>
<td>3.83 ± 0.90</td>
<td>4.51 ± 0.93</td>
<td>0.21</td>
<td>0.24</td>
</tr>
</tbody>
</table>

<sup>a</sup> The analyses were conducted in 2 different cohorts: the intention-to-treat cohort, which included all of the 96 participants, and the active-treatment cohort, which included the subjects who participated in the follow-up study and consumed ≥75% of the prescribed supplement during the intervention study. The term percentage gain refers to the percentage accretion in bone mass since the time of inclusion in the calcium-supplementation study (study A).

<sup>b</sup> Adjusted for the initial measurement at the time of inclusion.

<sup>c</sup> One-year double-blind calcium-supplementation study.

<sup>d</sup> x ± SEM (all such values).

<sup>e</sup> Follow-up study conducted 3.5 ± 0.7 y after the end of the intervention study.

---

**Table 5**

Regression analysis predicting accretion of total-body bone mineral density in the active-treatment cohort from baseline to 3.5 y after discontinuation of the intervention

<table>
<thead>
<tr>
<th>Variable</th>
<th>Standardized β coefficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (calcium vs placebo)</td>
<td>0.21</td>
<td>0.05</td>
</tr>
<tr>
<td>Time since baseline</td>
<td>0.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age at baseline</td>
<td>−0.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Weight at baseline</td>
<td>−0.42</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
In our study, at the end of study A (28), the calcium-supplemented group had a higher accretion of TBBMD than did the placebo group. In study B, TBBMD accretion from the time of inclusion in study A was still higher in the calcium-supplemented group. However, statistical significance was shown only for the active-treatment cohort, who had compliance rates of ≥75% during the intervention study (Table 3). Our findings suggest that after cessation of the calcium supplementation, the calcium-supplemented group did not show any further increases in bone mass. However, the positive effect of calcium supplementation on TBBMD accretion in study A in the active-treatment cohort did not disappear.

Another important result that supported this hypothesis was that attribution to the calcium-supplemented group in study A was found to be a statistically significant positive predictor of TBBMD accretion from baseline to study B in the active-treatment cohort. The results of our research suggest that the increase in BMD resulting from calcium supplementation for 1 y in postmenarcheal girls with low calcium intakes could be maintained >3 y after the end of the intervention. Consequently, our research tends to support the idea that calcium supplementation may provide a sustained effect on BMD and positively influence bone accretion during growth.

A possible reason for not achieving statistically significant results, as in the Bonjour et al study (27), was the difference in the pubertal stages of the participants in the 2 studies. In the study by Bonjour et al (27), the participants were premenarcheal girls with a mean age of 8 y. In our study, the participants were ≥1 y postmenarcheal with a mean age of 14 y. Puberty is a time of accelerated growth in which pronounced bone gain is achieved. Another possible reason was the low mean compliance rate in study A (67.33 ± 2.5%). This low compliance rate was probably a major obstacle for achieving significant results.

A great deal of effort was invested to evaluate the potential influence of other bone-modifying factors on bone mass acquisition during the period between the studies. The aim was to estimate whether the study and the control groups were exposed to the same factors that might alter bone mass and therefore affect the densitometric values. In the active-treatment and in the intention-to-treat cohorts, no significant differences were found between the study and the control groups.

Consequently the differences between the groups in bone mass accretion from the beginning of the calcium-supplementation study to the follow-up study arose from the primary distinction between the groups, ie, calcium supplementation in study A. This study suggests that 1 y of calcium supplementation in postmenarcheal girls with low calcium intakes had a sustained effect on total-body BMD, but no sustained effect was observed on the other skeletal areas. A possible explanation for this was that the permanent effect was present only in the cortical bone; therefore, the TBBMD that represents the cortical bone was the most important of the bone variables to be affected.

To the best of our knowledge, this study had the longest follow-up period for monitoring bone mass accumulation in postmenarcheal girls. It evaluated the net effect of calcium supplementation on bone mass in a population that had completed the rapid bone accretion that occurs during puberty.

The main conclusion of the study is that calcium supplementation of postmenarcheal girls with low calcium intakes for 1 y may provide a sustained effect on TBBMD accretion in girls with compliance rates of ≥75%. Longer-term calcium-supplementation studies in adolescents are necessary to confirm whether high calcium intakes can improve peak bone mass.

SI-S designed the study, obtained the research grant support from the Chief Scientist Fund of the Israeli Ministry of Health, and supervised RPD-G’s work and the writing of the manuscript. RPD-G conducted the field research and wrote the manuscript as part of the requirements for an MD thesis. GSR obtained the research grant support from the Rambam Medical Center Research Foundation, contributed to the fieldwork, and participated in the design of the study, the supervision of the research, and the writing of the manuscript. GR participated in the supervision of the research and the statistical analysis. HSR performed the statistical analyses. The authors had no financial or other interests relating to the study.

REFERENCES


Calcium supplementation and bone mineral density in females from childhood to young adulthood: a randomized controlled trial

Velimir Matkovic, Prem K Goel, Nancy E Badenhop-Stevens, John D Landoll, Bin Li, Jasminka Z Ilich, Mario Skagor, Larry A Nagode, Stacey L Mobley, Eun-Jeong Ha, Thomas N Hangartner, and Albert Clairmont

ABSTRACT

Background: Short-term studies established that calcium influences bone accretion during growth. Whether long-term supplementation influences bone accretion in young adults is not known.

Objective: This study evaluated the long-term effects of calcium supplementation on bone accretion among females from childhood to young adulthood.

Design: A 4-y randomized clinical trial recruited 354 females in pubertal stage 2 and optionally was extended for an additional 3 y. The mean dietary calcium intake of the participants over 7 y was ≈830 mg/d; calcium-supplemented persons received an additional ≈670 mg/d. Primary outcome variables were distal and proximal radius bone mineral density (BMD), total-body BMD (TBBMD), and metacarpal cortical indexes.

Results: Multivariate analyses of the primary outcomes indicated that calcium-supplementation effects vary over time. Follow-up univariate analyses indicated that all primary outcomes were significantly larger in the supplemented group than in the placebo group at the year 4 endpoint. However, at the year 7 endpoint, this effect vanished for TBBMD and distal radius BMD. Longitudinal models for TBBMD and proximal radius BMD, according to the time since menarche, showed a highly significant effect of supplementation during the pubertal growth spurt and a diminishing effect thereafter. Post hoc stratifications by compliance-adjusted total calcium intake and by final stature or metacarpal total cross-sectional area showed that calcium effects depend on compliance and body frame.

Conclusions: Calcium supplementation significantly influenced bone accretion in young females during the pubertal growth spurt. By young adulthood, significant effects remained at metacarpals and at the forearm of tall persons, which indicated that the calcium requirement for growth is associated with skeletal size. These results may be important for both primary prevention of osteoporosis and prevention of bone fragility fractures during growth. Am J Clin Nutr 2005;81:175–88.

KEY WORDS Calcium, growth, skeletal development, peak bone mass, osteoporosis, females

INTRODUCTION

Peak bone mass is one of the main determinants of osteoporotic fracture in humans (1); it is strongly influenced by genetics, but it could also be related to nutrition and exercise (2). We suggested previously that calcium might be an important determinant of peak bone mass in young adults by influencing bone accretion during growth (1, 3, 4). All clinical trials with calcium supplements in children and adolescents completed to date were relatively short and showed a positive effect of intervention on bone mass in young persons (3, 5–10). However, the extent to which those benefits could be translated to skeletal maturity was not confirmed. To understand further the nutritional determinants of peak bone mass, we conducted a long-term study with calcium supplementation in a cohort of females from childhood to young adulthood. The 7-y study included phases of bone modeling (change in size and geometry) during the pubertal growth spurt and of bone consolidation (endosteal apposition) during late adolescence, which represent the periods before and after epiphyseal closure, respectively.

SUBJECTS AND METHODS

Participants and sample size

The study was conducted in a cohort of young females recruited from 20 school districts in central Ohio. The minimum sample size calculation, based on the forearm bone mineral density (BMD) measurements in a pilot study (3), showed that 105 subjects per group were required to detect a 2.7% difference in BMD at the proximal radius by the end of the second year at α = 0.05, with power ≥ 0.8. An invitation form, a food-frequency
A questionnaire, and a pubertal stage self-report form were mailed to 15,000 female students aged 8–13 y (11). The inclusion criteria were white race, normal physical and mental health, pubertal stage 2, and calcium intake (determined by food frequency) below the threshold level of 1480 mg/d (12). The exclusion criteria were a history of metabolic bone, kidney, liver, or celiac disease; use of oral cortisone, hormones, diuretics, or antiseizure medications; other current systemic, chronic disease; and the presence of clinically significant abnormal laboratory data on screening. Response rates of 8–20% across schools generated initial data on 2000 girls who were willing to participate in the study. From this screening sample, 354 persons satisfied the inclusion criteria and were recruited for the study (Figure 1).

The study protocol was approved by the Biomedical Sciences Institutional Review Board at The Ohio State University. All minors and their parents gave written informed consent.

**Study protocol**

This study was originally designed as a randomized, double-blind, 4-y controlled clinical trial to assess the effect of calcium citrate-malate supplementation (1000 mg/d, given in 4 pills) on
the BMD of the total body, the radius, and the metacarpal radiographic measurements of adolescent females during the pubertal growth spurt. The study was subsequently extended for 3 y, into late adolescence, in the subjects who agreed to continue; double-blind status was preserved. On the basis of our previous cross-sectional study (13), participants were first separated into 4 strata with the use of baseline total-body BMD (TBBMD) and body mass index (BMI; in kg/m²), each at 2 levels (above and below the average value). The characteristics of the subjects were TBBMD < 0.879 and BMI < 18.5 in stratum A (n = 122), TBBMD < 0.879 and BMI ≥ 18.5 in stratum B (n = 45), TBBMD ≥ 0.879 and BMI < 18.5 in stratum C (n = 79), and TBBMD ≥ 0.879 and BMI ≥ 18.5 in stratum D (n = 108). The purpose of stratification was to allow for the equal distribution of bone measurements between the placebo and supplemented groups. Within each stratum, subjects were randomly assigned to either group. A list of consecutive random assignments to the calcium supplementation and placebo groups within each stratum, prepared by a statistician, led to 177 subjects within each group. A simple coding system linked the drug packages to the randomization list. The Proctor & Gamble Company provided calcium citrate-malate and placebo (microcrystalline cellulose) pills whose palatability and appearance were equal. Subjects were given a 6-mo supply of pills at each visit; additional pills were mailed if a return appointment had to be postponed. Subjects were instructed to take 2 pills in the morning and 2 pills in the evening. Compliance was monitored by pill counts and assessed by fecal calcium density, serum parathyroid hormone (PTH) concentrations, and 24-h urinary calcium excretion.

Outcome measures and confounding factors

Primary outcome measures in this study were bone mineral areal density of the whole body and radius at 2 sites (proximal (33% of the radius length) and distal (10% of the radius length)), and metacarpal cortical index. Metacarpal radiogrammetry was previously used in the study of nutrition, peak bone mass, and hip fracture rates in adults (1) and was shown to distinguish children with upper-limb fractures from their control subjects (14). Secondary outcome measures in the current study were stature, bone width, bone area, and bone turnover markers. Measures to control for confounding factors included assessment of nutritional status (ie, dietary calcium, protein, energy intake, and 24-h urinary sodium), energy expenditure, body weight, skeletal age, and pubertal development. Medical history, physical examination, and completed dietary and physical activity questionnaires and weight, height, and bone mass measurements of the whole body and the forearm were obtained at baseline and every 6 mo; blood (drawn between 0800 and 1700), 24-h urine, and stool samples (no baseline data) were obtained at baseline and annually. Hand X-rays for skeletal age and radiogrammetry of the metacarpal bones were obtained at baseline and at years 4 and 7. The subject’s weight was measured to the nearest 0.1 kg while the subject was wearing normal indoor clothing but no shoes. Standing height to the nearest 0.1 cm was recorded on a wall-mounted stadiometer (nearest 0.1 cm) while the subject was without shoes and with the mandible plane parallel to the floor (11). The subjects self-assessed their pubertal stage by marking on a chart the appropriate figures of sexual development, and the onset of menarche was documented within 6 mo of the event (15). Body composition (ie, lean body mass and body fat) and BMD were measured by using dual-energy X-ray absorptiometry (DXA; GE-Lunar DPX-L) with DPX-L software (version 1.3q; GE-Lunar). The precision errors (%CV) for the whole body and radius shaft BMD measurements were 0.5% and 0.8%, respectively (15). The two-person interobserver error for DXA analysis was < 0.1%. Daily phantom measurements on the DXA indicated a steady but extremely slow machine drift (a total decrease of 1.8% over 5 y); BMD was adjusted accordingly. Skeletal age was determined from radiographs of the nondominant hand by using the FELS method (16). Radiogrammetry of the metacarpals was performed by using the automated X-Posure System (Pronosco A/S, Vedboek, Denmark; 17). The CVs for the static and repositioned measurements were 0.0% and 0.2% for bone width and 0.5% and 0.6% for cortical thickness, respectively (18). Metacarpal cortical area (CA) and total cross-sectional area (TA) and the metacarpal cortical index, the ratio of CA to TA, were calculated. Nutritional status was assessed from 3-d dietary food records by using NUTRITIONIST III software (version 8.5; Hearst Corp, San Bruno, CA). Weight-corrected energy expenditure was estimated by recording activity in 15-min intervals for 2 d (11, 19). Serum urine and stool specimens were stored at −80 °C and −20 °C, respectively, and analyzed in multiple batches at different times throughout the study. Basic blood and urine chemistry measurements were made by using a Hitachi 717 Chemistry Analyzer (Boehringer-Mannheim, Indianapolis), and serum calcium was adjusted per total protein. Stool samples were collected before visits and aliquot used for fecal calcium density (mg calcium/g dry feces) measurement as previously described (20). Urine and fecal calcium were measured by atomic absorption spectrophotometry. Urinary N-telopeptides (NTX; nmol · L−1 · bone collagen equivalent/24 h) were measured by a competitive inhibition enzyme-linked immunosorbent assay (Osieomark; Ostex International, Seattle). Serum 25-hydroxyvitamin D3 ([25(OH)D3] was measured in each sample (excluding baseline) by using a radioimmunoassay (RIA) with 125I-labeled tracer (21). Serum osteocalcin was measured by immunoradiometric assays, and PTH was measured by Allegro immunoassay kits (both: Nichols Institute, San Juan Capistrano, CA). The stability of the PTH was shown by 99.1% agreement between repeated assays of a subsample (n = 10) in 1994 and 2000.

Statistical analysis

This long-term study allowed us to evaluate the effectiveness of calcium supplementation on bone mineral accretion during the period when most of the bone mass is accumulated. The distributions of confounding factors within the calcium-supplemented and placebo groups were compared by using the two-sample Kolmogorov-Smirnov tests for equality of distributions of average cumulative response over all visits for the 4- and 7-y cohorts. For the 7-y cohort, the distributions of average cumulative response over all visits during the first 4 y were also compared. The primary outcome variables were analyzed by using repeated-measures multivariate analysis of variance (MANOVA) (visits 1–9 or 1–15) consisting of main effects and interactions for treatment and visits, as well as a more general MANOVA (visits 2–9 or 2–15), in which the observations on the same person were treated as response variables with the use of an arbitrary variance-covariance matrix with baseline values as covariates. The gains from baseline were also analyzed by using repeated-measures MANOVA with baseline values as covariates. If the repeated-measures MANOVA indicated a significant interaction
for the primary and secondary outcome variables and calcium metabolic measurements. On the basis of bone biology, 4 knots at $-2, 0, +2,$ and $+4$ YSM were used in bone outcome models. For the bone turnover markers, serum calcium, serum PTH, and fecal and urinary calcium concentrations, which were collected yearly, 3 knots at $-2, 0,$ and $+2$ YSM were used. Because the randomization to the 2 treatment groups was performed within each of the 4 strata, our initial model for the $2 \times 2 \times 2$ factorial experiment included distinct regression splines for each subgroup (25). However, the factor “baseline BMI” was dropped from the model because it showed no significant effect on the bone accretion profiles. Furthermore, the factor “baseline TBBMD” had only a significant main effect ($\pm 0.038$, $P < 0.001$) in the TBBMD accretion patterns without any interaction with the pill or placebo factor. The models were fitted for different combinations of boundary knots, all of which provided qualitatively similar results. The results based on boundary knots at $-3$ YSM (baseline average $- SD$) and at $+6$ YSM (last visit average $+ SD$) are presented here.

The biologic efficacy (22, 23) of calcium intake was evaluated by using LME analysis of data after post hoc stratification of subjects based on the average total cumulative calcium intake over time (ie, above or below the habitual dietary calcium intake of 830 mg/d), irrespective of assigned group. Total calcium intake for subjects in the supplemented group included dietary calcium plus pill calcium after adjustment for compliance.

Finally, post hoc stratifications according to the final height or the TA above and below the median (ie, tall or short and larger or smaller bones, respectively) were also performed in subgroup analyses to establish whether body or bone size affects calcium requirement as previously indicated (33, 34). The two-factor (ie, group and size) ANCOVA for BMD at proximal radius and cortical bone mass (CA), with the baseline value of the response variable used as a covariate, was performed at both the year 4 and the year 7 endpoints to assess the overall differences among the 4 subgroups by using the F test. The contrast between the means of the placebo and calcium-supplemented groups for persons within each size subgroup was tested by using t tests to assess their interactions. We used S-PLUS 2000 for WINDOWS software (Professional Release 3; Insightful Corporation, Seattle) for all statistical analyses (35).

RESULTS

Study population

Participant flow is shown in Figure 1. Baseline characteristics of the study population are presented in Table 1. There were no significant differences at baseline in any of the values between the placebo and calcium-supplemented groups. Moreover, the cohorts with $\geq 2$ visits, those who completed the 4-y and 7-y intervention, and those in the high or low calcium intake subgroups did not differ significantly in baseline characteristics between the 2 groups. In addition, the distributions of the confounding factors, including pubertal stage, skeletal age, dietary proteins, energy intake and expenditure, and urinary sodium, did not differ significantly between the 2 groups throughout the study (range of $P$ values: 0.30–0.83). The average serum calcidiol concentration over time was $27.9 \pm 10.2$ mg/mL and $27.5 \pm 10.9$
ng/mL in the supplemented and placebo groups, respectively (range of \(P\) values: 0.33–0.55). The placebo group had a slightly higher proportion of ever-users of contraceptives (23%) than did the supplemented group (13%). However at age \(\approx 18\) y, the two-way (ie, group and contraceptive use indicator) ANOVA for the bone variables found no interaction between the factors TBBMD (\(P = 0.642\)), proximal radius BMD (\(P = 0.758\)), and CA:TA (\(P = 0.527\)).

The average dietary calcium intake among the study participants was \(830 \pm 236\) mg/d. This intake remained practically unchanged over the 7-y period and was considered the habitual dietary calcium intake of the study population (Table 2). The average total calcium intake in the supplemented group (1498 \(\pm 318\) mg/d) was close to the calcium intake threshold for adolescents (12). Total calcium intake in the supplemented group declined by \(\approx 10\%\) from age 15.5 y to age 18 y, primarily because of a decrease in compliance with pill taking (Table 2). In addition, several placebo group subjects had an average cumulative calcium intake comparable to the total calcium intake in the supplemented group.

### Compliance measures

The average cumulative compliance with pill taking during both the first 4-y period (70 \(\pm 20\%\) and 71 \(\pm 21\%\) for the supplemented and placebo groups, respectively) and the 7-y period (65 \(\pm 22\%\) and 66 \(\pm 22\%\)) did not differ significantly between groups. The calcium-supplemented group had significantly higher fecal calcium density (\(P < 0.001\)), higher serum calcium concentrations (\(P < 0.009\)), lower serum PTH concentrations (\(P < 0.001\)), and higher urinary calcium excretion (\(P < 0.006\) (Figure 2) than did the placebo group. PTH concentrations in the placebo group reached their peak by the onset of menarche and declined thereafter, subsequent to changes in serum calcium. Fecal calcium density and urinary calcium were
lower in both groups during the premenarchal period than after menarche. The trend was not clinically significant, but the fact that the pattern changes at menarche is clinically significant.

**Bone turnover markers**

For serial measurements of the markers of bone formation (ie, serum alkaline phosphatase and osteocalcin) and bone resorption (urinary NTX), the YSM was the best descriptor of the events associated with puberty. The markers all reached their peak near the onset of menarche and declined thereafter. There were no significant differences in serum total alkaline phosphatase (P = 0.3), serum osteocalcin (ng/mL) (P = 0.69; Figure 3), and urinary NTX excretion (nmol/L bone collagen equivalent/d; P = 0.31; Figure 3) between supplemented and placebo groups.

**Stature, bone width, and bone mineral area**

After 4y of intervention there was no significant difference in gains in stature, TA, and bone mineral areas between the calcium-supplemented and placebo groups (Table 3). Nor were differences in gains in stature, metacarpal TA, and bone mineral areas confirmed after 7y of intervention (Table 4). In addition, a repeated-measure MANOVA for height, metacarpal TA, and bone mineral areas, in which baseline values were used as covariates, showed no significant group × time interactions or group main effects.

**Bone mineral density and metacarpal radiogrammetry**

Because the original clinical trial was set up for a 4-y duration, we first present analyses of the primary outcomes for this cohort, to dispel the possible idea that the extension of the trial may have been conducted to achieve significant results. The general MANOVA on primary outcome variables (visits 2–9) for the 4-y cohort, in which baseline values were used as covariates, and the general MANOVA for the gain from baseline (supplemented group: n = 100, placebo group: n = 120) indicated highly significant treatment effects on TBBMD (covariate analysis: P < 0.0003; gain: P < 0.0006). Similar analyses for proximal (supplemented group: n = 91, placebo group: n = 108) and distal (supplemented group: n = 93, placebo group: n = 111) radius BMD, in which baseline values were used as covariates, found significant effect for distal radius BMD only (P < 0.0026). The repeated-measures MANOVA for these 3 variables (visits 1–9) and for their gain from baseline (visits 2–9), in which baseline values were used as covariates, found that, for the BMD measurements, group × visits interactions are all highly significant (P < 0.028), but, for the gains from the baseline, none of these interactions are significant (P > 0.572). The repeated-measures MANOVA for metacarpal CA and CA:TA (supplemented group: n = 101, placebo group: n = 118), in which baseline values were used as covariates, found significant interaction effects for CA:TA but an insignificant interaction effect for CA (ratio of CA to TA: P < 0.02; CA: P > 0.09). The ANOVA for gain from baseline, in which baseline values were used as covariates, showed significant differences between the groups for CA:TA (P < 0.024). Table 3 shows the data for these variables at baseline and at the year 4 endpoint and their gains from the baseline, along with the follow-up univariate analyses for BMD at the 3 sites and for metacarpal CA and CA:TA. The follow-up univariate analyses of these data at the year 4 endpoint show that TBBMD, proximal and distal radius BMD, metacarpal CA:TA, and the gains in TBBMD and metacarpal CA:TA, when baseline measurements were used as covariates, were significantly higher in the calcium-supplemented group than in the placebo group.

For the 7-y cohort, the general MANOVA for comparing the mean responses on primary outcome variables (visits 2–15), in which their baseline values were used as covariates, found a highly significant treatment effect on TBBMD (supplemented group: n = 60; placebo group: n = 67; P < 0.0004), proximal radius BMD (supplemented group: n = 56; placebo group: n = 63; P < 0.024), and distal radius BMD (supplemented group: n = 57; placebo group: n = 64; P < 0.028). The repeated-measures

---

**Table 2**

Dietary and total calcium intake by age of subjects in calcium-supplemented and placebo groups

| Age (y) | Supplemented group | Placebo group | Dietary and total calcium intake by age of subjects in calcium-supplemented and placebo groups
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total calcium intake</td>
<td>Dietary calcium intake</td>
</tr>
<tr>
<td>11</td>
<td>855 ± 288 (134)</td>
<td>855 ± 288</td>
</tr>
<tr>
<td>11.5</td>
<td>1589 ± 373 (114)</td>
<td>859 ± 305</td>
</tr>
<tr>
<td>12</td>
<td>1569 ± 352 (103)</td>
<td>791 ± 284</td>
</tr>
<tr>
<td>12.5</td>
<td>1577 ± 330 (114)</td>
<td>809 ± 256</td>
</tr>
<tr>
<td>13</td>
<td>1584 ± 390 (111)</td>
<td>828 ± 287</td>
</tr>
<tr>
<td>13.5</td>
<td>1607 ± 430 (97)</td>
<td>890 ± 348</td>
</tr>
<tr>
<td>14</td>
<td>1560 ± 455 (99)</td>
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</tr>
<tr>
<td>14.5</td>
<td>1533 ± 399 (92)</td>
<td>853 ± 311</td>
</tr>
<tr>
<td>15</td>
<td>1544 ± 457 (96)</td>
<td>869 ± 379</td>
</tr>
<tr>
<td>15.5</td>
<td>1504 ± 500 (79)</td>
<td>908 ± 408</td>
</tr>
<tr>
<td>16</td>
<td>1336 ± 539 (79)</td>
<td>790 ± 331</td>
</tr>
<tr>
<td>16.5</td>
<td>1441 ± 528 (64)</td>
<td>844 ± 353</td>
</tr>
<tr>
<td>17</td>
<td>1396 ± 609 (69)</td>
<td>839 ± 391</td>
</tr>
<tr>
<td>17.5</td>
<td>1427 ± 588 (60)</td>
<td>873 ± 382</td>
</tr>
<tr>
<td>18</td>
<td>1296 ± 567 (79)</td>
<td>824 ± 351</td>
</tr>
</tbody>
</table>

All values are x ± SD; n in parentheses. Two-sample t test for the difference between the total calcium intake in the placebo and calcium-supplemented groups found no difference between the groups at average age 11y (baseline: P < 0.3) and a highly significant difference between the groups at each age level above age 11y (P < 0.0001). The difference between the dietary calcium intakes in the 2 groups was not significant at any age level.
MANOVA (visits 1–15), in which baseline values were used as covariates, found highly significant treatment \times visits interaction for all 3 variables \((P < 0.0006)\). The repeated-measures MANOVA of the gains from the baseline for this cohort, in which baseline measurements were used as covariates, found highly significant treatment \times visit interactions in TBBMD \((P < 0.0097)\) and proximal radius BMD \((P < 0.001)\). A subanalysis of gain from the baseline by using general MANOVA on the follow-up data from the first 4 y only (visits 2–9) found that the supplemented and placebo groups were significantly different (TBBMD: \(P < 0.0004\); proximal radius BMD: \(P < 0.004\)). However, the general MANOVA for the gains from baseline for the same cohort during the 3-y study extension (visits 10–15) did not find a difference between the groups (TBBMD: \(P < 0.220\); proximal radius BMD: \(P < 0.135\)).

**FIGURE 2.** Longitudinal patterns based on the linear mixed-effect (LME) model for the supplemented (—) and placebo (---) groups for biochemistry data with years since menarche and the corresponding 95% CIs for their difference. A and B: supplemented group, \(n = 106\); placebo group, \(n = 125\); C and D: supplemented group, \(n = 116\); placebo group, \(n = 137\); E and F: supplemented group, \(n = 115\); placebo group, \(n = 137\); G and H: supplemented group, \(n = 115\); placebo group, \(n = 137\). The CI provides the estimated range of values for years since menarche during which the 2 groups differ significantly; when the CI contains zero, the difference at that time point is not significant. FCD, fecal calcium density; PTH, parathyroid hormone.
proximal radius BMD: $P < 0.346$). The repeated-measures MANOVA for metacarpal CA and CA:TA, in which baseline values were used as covariates (visits 1, 9, 15; supplemented group: $n = 76$; placebo group: $n = 96$), found that treatment × visit interactions were significant for these variables (CA: $P < 0.024$; ratio of CA to TA: $P < 0.0034$). However, for the gains from baseline, when baseline measurements were used as covariates, the interaction was significant for CA:TA ($P < 0.0000$) but not for CA ($P > 0.11$). Table 4 provides data for these variables at baseline, at the year 4 and year 7 endpoints, and their gains from the baseline, along with the results of follow-up univariate analyses for BMD at the metacarpal CA and CA:TA.

**TABLE 3**

Stature and bone outcome measures in the 4-y cohort of subjects in the supplemented and placebo groups at baseline and year 4

<table>
<thead>
<tr>
<th></th>
<th>Baseline Group</th>
<th>Year 4 Group</th>
<th>Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supplemented</td>
<td>Placebo</td>
<td>Supplemented</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>145.1 ± 7.4</td>
<td>145.1 ± 7.0</td>
<td>163.2 ± 5.6</td>
</tr>
<tr>
<td>Total-body BMD (g/cm²)</td>
<td>0.893 ± 0.057</td>
<td>0.891 ± 0.059</td>
<td>1.108 ± 0.066</td>
</tr>
<tr>
<td>Total-body BMA (cm²)</td>
<td>1466 ± 170</td>
<td>1457 ± 180</td>
<td>2040 ± 167</td>
</tr>
<tr>
<td>Proximal radius BMD (g/cm²)</td>
<td>0.501 ± 0.047</td>
<td>0.498 ± 0.048</td>
<td>0.654 ± 0.048</td>
</tr>
<tr>
<td>Proximal radius BMA (cm²)</td>
<td>2.077 ± 0.192</td>
<td>2.084 ± 0.192</td>
<td>2.247 ± 0.195</td>
</tr>
<tr>
<td>Distal radius BMD (g/cm²)</td>
<td>0.280 ± 0.039</td>
<td>0.276 ± 0.040</td>
<td>0.386 ± 0.054</td>
</tr>
<tr>
<td>Distal radius BMA (cm²)</td>
<td>2.248 ± 0.261</td>
<td>2.228 ± 0.231</td>
<td>2.655 ± 0.237</td>
</tr>
<tr>
<td>Calcium (cm²)</td>
<td>0.136 ± 0.020</td>
<td>0.133 ± 0.017</td>
<td>0.186 ± 0.023</td>
</tr>
<tr>
<td>TA (cm²)</td>
<td>0.392 ± 0.052</td>
<td>0.393 ± 0.051</td>
<td>0.436 ± 0.052</td>
</tr>
<tr>
<td>CA:TA</td>
<td>0.348 ± 0.039</td>
<td>0.341 ± 0.037</td>
<td>0.427 ± 0.042</td>
</tr>
</tbody>
</table>

1. All values are $\bar{x}$ ± SD. BMD, bone mineral density; BMA, bone mineral area; CA, metacarpal cortical area; TA, metacarpal total area. Univariate two-sample $t$ test for gain and analysis of covariance for the placebo ($n = 121$) and supplemented ($n = 101$) groups, with baseline values as covariate, at the 4-y endpoint, whenever the repeated-measures multivariate analysis of variance (MANOVA) indicated a significant interaction effect or the general MANOVA indicated significant differences in the mean responses. Because the MANOVAs ignore a case with even one missing observation, the number of subjects reported in the text for some of the MANOVAs was substantially less than the number of subjects reported in the table, which includes all subjects who were measured at the endpoint.

2. $P < 0.01$, 3$P < 0.05$. 

Figure 3. Longitudinal patterns based on the linear mixed-effect (LME) model for the supplemented (—) and placebo (—) groups for bone turnover biomarkers with years since menarche and the corresponding 95% CIs for their difference. A and B: supplemented group, $n = 116$; placebo group, $n = 137$; C and D: supplemented group, $n = 115$; placebo group, $n = 137$. The differences between the groups were not significant for osteocalcin and urinary N-telopeptide. The CI provides the estimated range of values for years since menarche during which the 2 groups differ significantly; when the CI contains zero, the difference at that time point is not significant.
TABLE 4  
Stature and bone outcome measures of the 7-y cohort of subjects in the supplemented and placebo groups at baseline and at years 4 and 7

<table>
<thead>
<tr>
<th></th>
<th>Baseline Group</th>
<th>Year 4 Group</th>
<th>Gain at 4 y Group</th>
<th>Year 7 Group</th>
<th>Gain at 7 y Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supplemented</td>
<td>Placebo</td>
<td>Supplemented</td>
<td>Placebo</td>
<td>Supplemented</td>
</tr>
<tr>
<td>Height (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supplemented</td>
<td>145.7 ± 7.3</td>
<td>144.7 ± 7.0</td>
<td>163.4 ± 5.5</td>
<td>163.2 ± 6.1</td>
<td>17.7 ± 4.8</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total-body BMD (g/cm²)</td>
<td>0.892 ± 0.055</td>
<td>0.888 ± 0.057</td>
<td>1.105 ± 0.067</td>
<td>1.094 ± 0.070</td>
<td>0.213 ± 0.037</td>
</tr>
<tr>
<td>Total-body BMA (cm²)</td>
<td>1475 ± 171</td>
<td>1444 ± 178</td>
<td>2043 ± 172</td>
<td>2022 ± 205</td>
<td>568 ± 122</td>
</tr>
<tr>
<td>Proximal radius BMD (g/cm²)</td>
<td>0.502 ± 0.046</td>
<td>0.496 ± 0.049</td>
<td>0.654 ± 0.049</td>
<td>0.641 ± 0.053</td>
<td>0.152 ± 0.029</td>
</tr>
<tr>
<td>Proximal radius BMA (cm²)</td>
<td>2.071 ± 0.197</td>
<td>2.079 ± 0.197</td>
<td>2.240 ± 0.206</td>
<td>2.239 ± 0.195</td>
<td>0.166 ± 0.090</td>
</tr>
<tr>
<td>Distal radius BMD (g/cm²)</td>
<td>0.279 ± 0.039</td>
<td>0.273 ± 0.040</td>
<td>0.387 ± 0.058</td>
<td>0.366 ± 0.058</td>
<td>0.108 ± 0.050</td>
</tr>
<tr>
<td>Distal radius BMA (cm²)</td>
<td>2.252 ± 0.261</td>
<td>2.230 ± 0.231</td>
<td>2.646 ± 0.240</td>
<td>2.656 ± 0.265</td>
<td>0.394 ± 0.161</td>
</tr>
<tr>
<td>CA (cm²)</td>
<td>0.137 ± 0.020</td>
<td>0.132 ± 0.017</td>
<td>0.186 ± 0.024</td>
<td>0.178 ± 0.021</td>
<td>0.049 ± 0.013</td>
</tr>
<tr>
<td>TA (cm²)</td>
<td>0.392 ± 0.053</td>
<td>0.392 ± 0.049</td>
<td>0.436 ± 0.053</td>
<td>0.434 ± 0.049</td>
<td>0.042 ± 0.018</td>
</tr>
<tr>
<td>CA:TA</td>
<td>0.349 ± 0.036</td>
<td>0.339 ± 0.035</td>
<td>0.429 ± 0.040</td>
<td>0.412 ± 0.047</td>
<td>0.079 ± 0.020</td>
</tr>
</tbody>
</table>

All values are x ± SD. BMD, bone mineral density; BMA, bone mineral area; CA, metacarpal cortical area; TA, metacarpal total area. Univariate two-sample t test for gain and analysis of covariance for the placebo (n = 98) and supplemented (n = 79) groups, with baseline values as covariate, at the 4-y and 7-y endpoints, whenever the repeated-measures multivariate analysis of variance (MANOVA) indicated a significant interaction effect or the general MANOVA indicated significant differences in the mean responses. Because the MANOVAs ignore a case with even one missing observation, the number of subjects reported in the text for some of the MANOVAs was substantially less than the number of subjects reported in the table, which includes all subjects who were measured at the endpoint(s).

² Significantly different from baseline: ²P < 0.05, ²²P < 0.01.
adjusted for the baseline values. The differences between the supplemented and placebo groups in TBBMD and distal BMD, which were significant at the year 4 endpoint, were no longer significant at the year 7 endpoint. In addition, the metacarpal CA and CA:TA and their gains from baseline were significantly higher in the supplemented group than in the placebo group at both these endpoints (Table 4). Collectively, these analyses clearly indicated a need for further examination of the longitudinal behavior of bone mass accretion in the supplemented and placebo groups.

The longitudinal analysis based on the LME model revealed a significant influence of calcium supplementation on TBBMD \((P < 0.001)\) and proximal radius BMD \((P < 0.001)\) (Figure 4).

The calcium-supplemented group showed a faster rate of bone mass accretion from the beginning of the study; the maximum difference between the BMD of the 2 groups at both of these sites occurred during the interval from \(-1\) YSM to \(+1\) YSM. After \(+1\) YSM, the differences between the groups started to diminish, and they became insignificant after \(+3\) and \(+5\) YSM for TBBMD and proximal radius BMD, respectively (Figure 4).

A post hoc stratification of subjects based on the compliance-adjusted average cumulative total calcium intake, irrespective of assigned group, showed significant influence of calcium on the proximal radius BMD pattern \((P < 0.0001)\) (Figure 5). The high-calcium-intake subgroup (average intake: \(1353 \pm 342\) mg/d; \(n = 180\)) has persistently higher BMD of the proximal radius

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**FIGURE 4.** Longitudinal patterns based on the linear mixed-effect (LME) model for the supplemented (—) and placebo (-----) groups for bone mineral density (BMD) with years since menarche and the corresponding 95% CIs for their difference. A and B: supplemented group, \(n = 115\); placebo group, \(n = 137\); C and D: supplemented group, \(n = 115\); placebo group, \(n = 137\). Gray background in panels A and C shows patterns of 15 randomly selected persons in each group. The CI provides the estimated range of values for years since menarche during which the 2 groups differ significantly; when the CI contains zero, the difference at that time point is not significant.

**FIGURE 5.** Longitudinal patterns based on the linear mixed-effect (LME) model for the post hoc strata with high (—) and low (-----) calcium intakes, irrespective of assigned group, showed significant influence of calcium on the proximal radius bone mineral density (BMD) with years since menarche and the corresponding 95% CIs for their difference: high-calcium-intake subgroup, \(n = 180\) (average cumulative calcium intake: \(1353 \pm 342\) mg/d); low-calcium-intake subgroup, \(n = 89\) (average cumulative calcium intake: \(668 \pm 118\) mg/d). The CI provides the estimated range of values for years since menarche during which the 2 groups differ significantly; when the CI contains zero, the difference at that time point is not significant.
from puberty to young adulthood than does the low-calcium-intake subgroup (average intake: 668 ± 118 mg/d; n = 89).

Skeletal size and calcium requirement

According to the two-factor (ie, pill or placebo and short or tall) ANCOVA of the proximal radius BMD, the overall F test indicated a significant effect (P < 0.04) at the year 4 endpoint and an insignificant effect (P = 0.23) at the year 7 endpoint. At year 4, the interaction between these 2 factors showed that, for taller subjects, the contrast between the placebo and calcium-supplemented groups was significant (P < 0.006), whereas, for shorter subjects, the test for the contrast did not reveal any significant effect (P = 0.826). Consequently, we performed separate LME analysis for the tall and short subgroups, which found a significant influence of calcium supplementation on bone mineral accretion at proximal radius in tall persons. The maximum difference between the BMD of the 2 groups was during the interval 1 YSM. After 5 YSM, the differences between the groups started to diminish and became insignificant after 5.5 YSM (Figure 6). Unlike the tall subgroup, the short subgroup did not show an effect of calcium supplementation on the proximal radius BMD (Figure 6).

The F test in the two-factor (ie, pill or placebo and larger or smaller bones) ANCOVA of the metacarpal CA found significant overall effects at both the year 4 (P < 0.006) and the year 7 (P < 0.005) endpoints. The interaction between these 2 factors showed that, among subjects with larger metacarpals, the contrasts between the placebo and calcium-supplemented groups were highly significant at both the year 4 (P < 0.02) and year 7 (P < 0.002) endpoints; however, the effect was not present among subjects with smaller metacarpals at either year 4 (P = 0.79) or year 7 (P = 0.70). This implies that a calcium intake of ≈830 mg/d was adequate for persons with smaller metacarpals but insufficient for those genetically predetermined to develop larger bones.

Bone fracture

Twenty girls from the placebo group and 9 girls from the calcium-supplemented group reported having a bone fracture because of a moderate trauma during the trial. There were 3 forearm fractures in the supplemented group (33%) and 11 in the placebo group (48%). The average timing of the fracture was +1.2 ± 0.4 YSM, which coincides with the bone-modeling phase in skeletal development and overlaps with the timing of the maximal effect of calcium supplementation on BMD among young females in this study (Figure 4).

DISCUSSION

This study documents that calcium supplementation (≈670 mg/d) beyond a habitual dietary calcium intake of ≈830 mg/d (increasing total calcium intake up to ≈1500 mg/d) positively influences bone mass acquisition throughout the bone-modeling phase of the pubertal growth spurt. This effect diminishes during the skeletal consolidation of late adolescence that is due to the catch-up phenomenon in bone mineral accretion. At the beginning of young adulthood, the positive effect of calcium supplementation was evident at all skeletal regions of interest; however, the only differences that remained significant were those at the metacarpals and at the proximal radius among the subgroup with high calcium intake and in tall persons.
The explanation for the catch-up phenomenon lies within the skeletal physiology of growth, which dictates calcium requirement (4, 12). Calcium requirement is the highest during the pubertal growth spurt, when most of the retained calcium contributes to skeletal build-up to accommodate longitudinal bone growth and periosteal bone expansion (4, 13). During this interval, ≈37% of the entire adult skeletal mass is accumulated (13). Therefore, inadequate calcium intake during this period compromises the bone mineral accretion rate (3). During the pubertal growth spurt, there was an obvious discrepancy between optimal and actual calcium intakes among the placebo group in this study, which was possibly reflected in a higher fracture rate, even though that was not a research outcome variable. However, when bone modeling slows down (by epiphyseal closure that results from increased estradiol secretion after menarche), the demand for calcium declines, and the calcium intake threshold decreases from 1480 to 957 mg/d (12). The habitual calcium intake in the young women in the current study is very close to this reduced requirement and is sufficient to accommodate bone consolidation, which allows BMD to catch up slowly.

These conclusions are supported by the blood, stool, and urine chemistry measurements. During bone modeling of the pubertal growth spurt, the PTH concentrations in the placebo group reached a maximum and declined thereafter. The increase in PTH could increase intracortical remodeling and porosity as indicated previously (36) and is reflected in a slightly increased urinary NTX excretion. The pubertal growth spurt is the time when serum calcitriol concentration is at its maximum and is increasing calcium absorption to accommodate skeletal needs (37), which results in a lower fecal calcium excretion (4), and presumably in a lower fecal calcium density. Calcium absorption decreases during bone consolidation in late adolescence (4), and fecal calcium density increases concomitantly. Calcium supplementation during the pubertal growth spurt partially blunts PTH secretion, which suggests that calcium intake is adequate, and bone resorption decreases concomitantly, as previously documented by calcium kinetics (38). The changes in serum PTH reflect the fluctuations in serum calcium concentration during growth. Serum calcium is lower during the bone-modeling phase of the pubertal growth spurt than it is either before that phase or later, during the bone-consolidation phase. Urinary calcium follows those changes closely (4). Urinary calcium excretion is lower during the bone-modeling phase than during skeletal consolidation. The difference in urinary calcium excretion between the 2 groups was much smaller during the pubertal growth spurt (7%) than it was in late adolescence (18%), which implies that urinary calcium is less dependent on intake during bone modeling, as documented by balance studies (3, 4). Calcium supplementation increased urinary calcium more during bone consolidation, despite a drop in calcium intake (by 10%), which suggests that the calcium supply is adequate.

The catch-up phenomenon in bone mass acquisition suggests the existence of a reversible mineral deficit that is acquired during the pubertal growth spurt (36) and that can be repaired during bone consolidation. The catch-up phenomenon was apparent, in particular at the total body. However, at the metacarpals and at the proximal forearm of persons accustomed to different calcium intakes, the difference created during bone modeling was maintained into young adulthood, which implies incomplete catch-up and a lower peak bone mass. The lower the habitual calcium intake in the population, the higher the possibility of a low peak bone mass at skeletal maturity. The results of ecologic studies (1, 39) conducted among populations accustomed to very low lifetime calcium intakes point in this direction. Metacarpal radiogrammetric measurements (ie, CA and CA:TA) were lower in women from a low-calcium-intake area of Croatia, who were accustomed to calcium intakes of ≈400 mg/d over a lifetime and who had a concomitantly high rate of hip fracture (1). Similar findings with regard to peak bone mass were obtained in animal experimentations by manipulating calcium in the diet early in life (40, 41).

In addition to BMD, the current study evaluated the effect of calcium supplementation on longitudinal (height) and periosteal (width) bone expansion and on bone mineral area. The results show that calcium supplementation in addition to the habitual calcium intake of ≈830 mg/d had no effect on bone geometry measurements.

To ascertain whether BMD is related to calcium supplementation among persons of different body frames, its interactions with final height and periosteal expansion (TA) were examined (33, 34). Among subjects destined to be taller or to have larger bones, those with higher calcium intakes had significantly higher BMD than did those with lower calcium intakes. These results strongly support the notion that dietary calcium requirement for skeletal development is size dependent.

All clinical trials with calcium or dairy product supplementation in children and adolescents that have been completed to date (3, 5–10, 42–44) showed a positive effect of intervention on bone mass, but they were all too short (1–3 y) to address the question of whether it is the adaptation of bone tissue to nutritional challenge that leads to peak bone mass. The increase in bone mass observed in those short-term studies could be explained to a large extent by the phenomenon of the bone-remodeling transient (45). In some of the studies reported earlier, the difference in bone mass between the groups diminished after calcium intervention was discontinued (46, 47), which indicated that the bone accretion gained in the first transient was lost as a result of the second (45). In other studies, the effects of intervention were maintained 1–3 y after discontinuation of treatment (44, 48, 49); this result may be specific to the calcium source, to the amount of habitual dietary calcium intake, or both.

In the current study, however, calcium supplementation continued without interruption for 7 y, which allowed for the adaptation in bone behavior during the bone-modeling and bone-consolidation phases in skeletal development and extended long after the first bone-remodeling transient effect ended (≈12 mo after the beginning of intervention). According to calcium balance studies (4), the calcium requirement of the participants during the 7-y follow-up was changing as well. The effect of the second transient, following the discontinuation of calcium supplementation after 7 y, therefore is not expected because dietary calcium requirements have been reduced to an amount closer to habitual dietary calcium intake. Thus, if we assume that these women will maintain their dietary habits, it is unlikely that the differences in bone mass measurements observed at these skeletal regions of interest at the beginning of young adulthood will disappear by the cessation of intervention. A follow-up study may be necessary for confirmation.

Some studies indicate that calcium intake may play a role in the prevention of fractures due to bone fragility during growth (50, 51). The fracture rate was not a primary research outcome, but the reported fracture incidence in our study points in this direction,
although the sample size may be too small for adequate interpretation. Because the peak incidence of fractures due to bone fragility coincides with the pubertal growth spurt (52, 53), our results indicate that calcium intakes at threshold level may reduce the risk of fracture during this stage of skeletal development, irrespective of the catch-up phenomenon in bone mass acquisition that occurs thereafter. The possibility that calcium intakes may reduce the risk of fracture is particularly important given the large number of childhood forearm fractures and the rising incidence over the last 30 y (54). A long-term intervention study of calcium supplementation in children that evaluates the forearm fracture as the main research outcome should be conducted to resolve this issue.

It is of great interest to pediatricians to tailor the nutritional recommendations for adolescents to their individual calcium needs, which are maximal at the pubertal growth spurt. However, such specification may be difficult to implement in practice because a person’s rates of bone growth and skeletal maturity cannot be predicted precisely. The results of the current study also have implications for dietary calcium intake standards for children and adolescents worldwide; the standards for one ethnic group might not be suitable for another. Each country should develop its own standards that are specific to the people living in the region. Factors such as ethnicity, dietary habits (including salt intake), sunlight exposure, and activity level all play a role, but stature and bone frame must also be considered.

In summary, this study documents that calcium supplementation in excess of a habitual calcium intake of ≈830 mg/d affects BMD during the pubertal growth spurt, but there is a diminishing effect thereafter that is due to the catch-up phenomenon in bone mineral accretion. By young adulthood, significant effects of calcium supplementation were present at metacarpals and at the proximal forearm in subjects who had better calcium compliance and in subjects who developed larger body frames. These results imply that standards for dietary calcium intake in adolescence should be based on growth rate and body and bone size development. The results of this study may be important for the prevention of bone fragility fractures during growth, as well as for the primary prevention of osteoporosis.

We are indebted to the participants in the study and to their parents and grandparents for continuous cooperation and enthusiasm that helped us to create a family project; to all the School Districts in Franklin County for helping us to create a school project; to the nursing and technical staffs of the General Clinical Research Center at The Ohio State University for their skilled and dedicated care in the collection of calcium metabolic data; to David G Cornwell and Manuel Trzagournis from the College of Medicine at The Ohio State University for their help with recruitment and study support; to Mark B Andon, Charles Schuster, and Kenneth T Smith from Procter & Gamble Company, and Douglas B DiRienzo from National Dairy Council for their help with the study; to Alex F Roche from Fells Institute for instructions in standard techniques and DXA. Finally, we thank the AJCN Manuscript Editor for many helpful suggestions that improved the overall presentation.

VM, TNH, and PKG were involved in the study design and study conduct; NEBS, JDL, JZI, MS, LAN, SLK, and EHJ were involved in various phases of data collection; PKG and BL performed the statistical analysis; TNH was involved in designing and implementing quality control for the DXA measurements; NEBS, JZI, and JDL were involved in database development and maintenance; VM, PKG, BL, AC, JDL, and JZI were involved in data analysis; VM and PKG had a major role in writing the manuscript; and all authors contributed to the writing of the manuscript. All authors have declared that no conflicts of interest exist.

REFERENCES
Randomized controlled trial of the effects of soy protein containing isoflavones on vascular function in postmenopausal women1–3

Sanne Kreijkamp-Kaspers, Linda Kok, Michiel L Bots, Diederick E Grobbee, Johanna W Lampe, and Yvonne T van der Schouw

ABSTRACT

Background: The incidence of cardiovascular disease increases after menopause, possibly because of the decline in estrogen. Soy protein, a rich source of estrogen-like isoflavones, is hypothesized to improve vascular function.

Objective: The objective of this study was to investigate whether supplementation with soy protein, a rich source of estrogen-like isoflavones, improves vascular function.

Design: We performed a 12-mo double-blind randomized trial to compare the effects of soy protein containing 99 mg isoflavones/d (aglycone weights) with those of milk protein (placebo) on blood pressure and endothelial function in 202 postmenopausal women aged 60–75 y.

Results: Changes in endothelial function during the intervention were not significantly different between the soy and the placebo groups. After the intervention, systolic blood pressure increased in the soy group significantly more than it did in the placebo group; the difference in change was 4.3 mm Hg (95% CI: 0.3, 8.4 mm Hg; \(P = 0.04\)) for systolic blood pressure, but only 2.0 mm Hg (95% CI: \(-0.7, 4.7\) mm Hg; \(P = 0.15\)) for diastolic blood pressure. In the soy group only, systolic and diastolic blood pressure decreased and endothelial function improved in the equol producers, whereas systolic and diastolic blood pressure increased and endothelial function deteriorated in the equol nonproducers.

Conclusions: The results of this trial do not support the hypothesis that soy protein containing isoflavones have beneficial effects on vascular function in older postmenopausal women. Whether certain subgroups of women (eg, equol producers) do benefit from the intervention remains to be elucidated. Am J Clin Nutr 2005;81:189–95.

KEY WORDS Isoflavones, soy, endothelial function, blood pressure, hypertension, postmenopausal women

INTRODUCTION

Cardiovascular disease is a major health concern in Western societies, and in women its incidence increases after menopause. This observation might be related to the sharp decline in endogenous estrogen production after menopause.

Isoflavones are estrogen-like compounds that are present in plant foods such as soy, nuts, and beans. A higher dietary soy intake has been reported to be associated with decreased cardiovascular mortality in Japanese women (1), but the consumption of soy and its associated isoflavones is 10–40-fold higher in Asian countries than in Western countries (2, 3). Even at the low levels of consumption that are normal in Western countries, higher usual dietary isoflavone intakes were associated with decreased aortic stiffness (4). The trials published thus far are inconclusive; some trials have found improvements in endothelial function with isoflavones (5–7), whereas others have reported no changes (8–11). Most of these studies were short in duration and enrolled small numbers of women.

Our aim was to investigate whether soy protein–containing isoflavones could improve endothelial function and blood pressure in postmenopausal women. We performed a 1-y double-blind, randomized, placebo-controlled trial with soy protein in 202 postmenopausal women.

SUBJECTS AND METHODS

Subjects

The subjects were identified via the database of a breast cancer screening program in Utrecht. Between March 2000 and September 2000 we randomly assigned 202 women in the trial. Details of the study were published previously (12). We excluded women with conditions for which estrogens are contraindicated, women with an endometrial thickness \(< 4\) mm, current and recent (within past 6 mo) estrogen users, and women with a known allergy or hypersensitivity to soy or cow milk. The Institutional Review Board of the University Medical Center Utrecht approved the study protocol, and all participants gave informed written consent.

Randomization and blinding

After completing the baseline tests, the subjects were randomly assigned to the intervention or the placebo group in blocks.
of 10. A list of randomization numbers was computer generated. Each randomization number corresponded with 1 of the 2 possible interventions, and personnel not involved in the trial attached a label with the number to the identically looking boxes that contained soy or total milk protein. The research dietitian assigned randomization numbers to the subjects in order of enrollment into the trial. To assess the efficacy of blinding, the participants were asked at the end of the intervention to which group they thought they had been assigned to, the placebo or the soy group.

**Intervention**

The women were randomly assigned to receive either soy protein or total milk protein. The intervention consisted of 25.6 g isoflavone-rich soy protein containing 52 mg genistein, 41 mg daidzein, and 6 mg glycitein (aglycone weights) in 36.5 g soy-protein powder (The Solae Company, St Louis) that could be mixed with food or beverages. The placebo (total milk protein) was identically looking and tasting and contained the same nutrients. Extra vitamins and minerals were added to the supplement for both groups (vitamin B-2, B-6, B-12, and D; folic acid; and calcium). One supplement was taken daily for a total period of 12 mo.

A certified dietitian assessed the usual dietary pattern with the use of a food-frequency questionnaire at baseline and at the final visit. This questionnaire was validated (13) and marginally modified to capture dietary phytoestrogen intake. The dietitian counseled the participants on how to incorporate the supplement into their diets, and they provided recipes and suggestions. The participants were individually advised about which sources to decrease their protein intakes from to compensate for the extra protein intake from the supplement.

**Measurements**

At the physical examination we measured height (with the subjects shoeless) to the nearest 0.5 cm and weight to the nearest 0.5 kg. A blood sample was drawn, and total cholesterol, LDL cholesterol, HDL cholesterol, and triacylglycerol were measured. Blood pressure and heart rate were measured with a Dinamap (Critikon Corp, Tampa, FL) on the right arm with the subjects in the sitting position. Blood pressure was measured at the screening visit, at randomization, and during the final visit. The baseline value was calculated as the mean of the measurements taken at the screening and the randomization visits. Measurements taken at the screening, at randomization, and during the final visit. The baseline value was calculated as the mean of the measurements taken at the screening and the randomization visits. Waist circumference, just above the iliac crest, and hip circumference at the trochanter major (in cm) were measured to estimate upper body adiposity. From the health questionnaire we obtained information about age at menarche, age at menopause, history of oral contraceptive use, use of hormone replacement therapy, use of cholesterol-lowering and antihypertensive medications, and smoking history. Physical activity was determined through the validated Questionnaire on Mobility in Elderly (14).

**Brachial endothelial function**

Endothelial function was measured with B-mode ultrasound imaging of the brachial artery by assessing the increase in artery diameter during reactive hyperemia (with increased flow leading to endothelium-dependent dilatation as detailed elsewhere) (15, 16).

In short, while the participants were in the supine position, a pediatric blood pressure cuff was positioned around the right forearm just below the elbow and where the electrocardiogram electrodes were placed. Blood pressure was measured. The participants were instructed not to talk during the measurements. With an ultrasound probe (7.5 MHz; Acuson Aspen, Mountain View, CA), the brachial artery was visualized. When a satisfactory longitudinal image of the brachial artery was obtained, the position of the transducer was secured. Three B-mode images showing the lumen diameter were frozen on the R-wave of the electrocardiogram for offline measurement of the baseline lumen diameter. Subsequently, the blood pressure cuff was inflated up to 50 mm Hg above the participant’s systolic blood pressure. After 4 min, the cuff was deflated.

During the subsequent phase of reactive hyperemia, the image of the brachial artery was recorded every 15 s for 5 min. The images were digitalized and subsequently analyzed with dedicated software (17). The dilatation was measured as the increase in diameter relative to the baseline diameter. The following equation gives the percentage change in diameter during the reactive hyperemia: (maximum diameter during reactive hyperemia − mean diameter)/mean diameter × 100. One person, blinded to the intakes of the subjects, performed all of the analyses, and a random sample of 10% was analyzed twice to assess reproducibility of the analyses. The Spearman correlation coefficient for repeated analyses was 0.81. In previous studies performed in our Vascular Imaging Center, in which the same technique used to measure flow-mediated dilatation (FMD) the SD within subjects was 2.6% FMD with a corresponding CV of 49%. The CV of resting and maximum diameter was 8% (15, 16, 18–20).

**Compliance**

Plasma genistein and equol concentrations were measured in the blood sample taken at the final visit with the use of TR-FIA kits (Labmaster, Turku, Finland) (21, 22). Fluorescence was measured with the use of the Victor 2 model 1420 spectrofluorimeter (Wallac, Turku, Finland). Data were analyzed by using GRAPHPAD PRISM software (GraphPad Software Inc, San Diego). Intraassay and interassay CVs were 2.2% and 14.8%, respectively. Subjects were considered to be equol producers if they had a plasma equol concentration >83 nmol/L (23). The proportion of equol producers was 29.9%. Equol-producer status could only be assessed within the soy group because an exposure to daidzein is required to determine equol status.

**Number of participants**

The planned number of subjects was 200, 100 in each intervention arm. This number was based on conventional assumptions of α = 0.05 and β = 0.20 and a withdrawal from intervention of 25% of the sample. Assuming furthermore that soy isoflavones are as effective as is conventional hormone replacement therapy, we would be able to demonstrate an improvement of 32% in FMD. Improvements of up to 70% in FMD have been reported for conventional estrogen replacement therapy (24, 25). The mean and SD values from studies in our own Vascular Imaging Center are comparable with those in the literature.

**Data analysis**

We performed a closeout visit when a subject had participated for ≥1 mo. Fourteen percent of the participants did not complete
RESULTS

Forty-nine (24%) participants did not complete the trial for various reasons; the most important reason being gastrointestinal complaints and aversion to the taste of the supplement. The median duration of participation in the dropouts was 79 d (range: 4–285 d). There was no significant difference in the dropout rate between the 2 intervention groups (n = 24 in the placebo group and 25 in the soy group).

The baseline characteristics of the participants, by intervention group, is shown in Table 1. Despite randomization, there were some differences at baseline. The number of current smokers was somewhat higher in the soy group (19.0%) than in the placebo group (12.7%), and the use of antihypertensive medication was significantly lower in the soy group (15.0%) than in the placebo group (28.4%). All medical complaints during the intervention were recorded as adverse events; the most common complaints were gastrointestinal symptoms, such as constipation or heartburn. The number of adverse events was not significantly different between groups; both groups reported 2.5 adverse events. There were also no significant differences in the types of adverse events reported. Analysis of the food-frequency questionnaires indicated no significant differences in dietary intake patterns between the 2 groups.

Both groups reduced their intakes of protein and total energy as a result of the nutrients provided by the supplement (Table 2). Women were asked whether they thought they were in the soy group or in the placebo group; the responses were not significantly different between the 2 groups (χ² = 0.75, P = 0.69), which confirmed that the blinding was effective. Genistein concentrations during the trial were significantly different between the soy and the placebo groups (1259 ± 1610 and 55 ± 101 nmol/L, respectively; P < 0.001), which demonstrated that compliance was good.

At baseline, both systolic and diastolic blood pressures were lower in the soy group than in the placebo group (systolic: 138 ± 16.7 and 143 ± 18.1 mm Hg, respectively; diastolic: 74 ± 10.7 and 76 ± 12.9 mm Hg, respectively). The other vascular indexes were not significantly different between groups at baseline. After the intervention, the systolic blood pressure tended to increase in the soy group relative to that in the placebo group; the difference

Table 1
Baseline characteristics of 202 postmenopausal women participating in the trial

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n = 102)</th>
<th>Soy (n = 100)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>66.8 ± 4.7</td>
<td>66.6 ± 4.8</td>
<td>0.75</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>69.5 ± 10.1</td>
<td>71.0 ± 11.3</td>
<td>0.33</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>76.1 ± 12.7</td>
<td>74.4 ± 10.8</td>
<td>0.30</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>142.8 ± 17.8</td>
<td>138.9 ± 16.7</td>
<td>0.11</td>
</tr>
<tr>
<td>Current use of antihypertensive medication [%]</td>
<td>29 (28.4)</td>
<td>15 (15.0)</td>
<td>0.02</td>
</tr>
<tr>
<td>Current use of cholesterol-lowering medication [%]</td>
<td>16 (15.7)</td>
<td>13 (13.0)</td>
<td>0.59</td>
</tr>
<tr>
<td>Brachial endothelial function [%FMD]</td>
<td>4.4 ± 3.4</td>
<td>4.8 ± 5.0</td>
<td>0.58</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.0 ± 3.4</td>
<td>26.4 ± 4.1</td>
<td>0.39</td>
</tr>
<tr>
<td>Time postmenopause (y)</td>
<td>17.9 ± 6.2</td>
<td>18.5 ± 7.6</td>
<td>0.52</td>
</tr>
<tr>
<td>Ever use of estrogens [%]</td>
<td>23 (22.5)</td>
<td>22 (22.0)</td>
<td>0.93</td>
</tr>
<tr>
<td>Smoking [%]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>13 (12.7)</td>
<td>19 (19.0)</td>
<td>0.23</td>
</tr>
<tr>
<td>Former</td>
<td>34 (33.3)</td>
<td>33 (33.0)</td>
<td>0.96</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>5.4 ± 1.6</td>
<td>5.5 ± 0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>6.1 ± 0.9</td>
<td>6.2 ± 1.2</td>
<td>0.6</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>4.1 ± 0.9</td>
<td>4.2 ± 1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.5 ± 0.3</td>
<td>1.5 ± 0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/L)</td>
<td>1.4 ± 0.8</td>
<td>1.3 ± 0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Lipoprotein(a) (g/L)</td>
<td>0.24 ± 0.28</td>
<td>0.29 ± 0.38</td>
<td>0.30</td>
</tr>
</tbody>
</table>

1 FMD, flow-mediated dilation.
2 ANOVA for continuous variables and chi-square test for categorical variables.
3 x ± SD (all such values).
in change was 4.3 (95% CI: 0.3, 8.4; \( P = 0.04 \)) mm Hg (Table 3). We repeated the analyses with baseline systolic and diastolic blood pressure as a covariate to adjust for baseline differences, but this did not change the results significantly (data not shown). When the women who were taking antihypertensive medication were excluded from the analyses, the differences were 3.8 (95% CI: −0.9, 8.5; \( P = 0.12 \)) mm Hg for systolic and 1.1 (95% CI: −1.5, 3.8; \( P = 0.41 \)) mm Hg for diastolic blood pressure (Table 3).

### TABLE 3
Effects of a soy supplement containing isoflavones on vascular function in postmenopausal women

<table>
<thead>
<tr>
<th>Intention to treat (n = 175)(^4)</th>
<th>Placebo group</th>
<th>Soy group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diastolic blood pressure</td>
<td>76.2 ± 12.9</td>
<td>74.5 ± 12.4</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>143.4 ± 18.2</td>
<td>139.9 ± 18.1</td>
</tr>
<tr>
<td>%FMD</td>
<td>4.4 ± 3.4</td>
<td>4.4 ± 4.0</td>
</tr>
<tr>
<td>Per protocol (n = 153)(^4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>75.7 ± 12.9</td>
<td>73.7 ± 12.2</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>143.4 ± 18.7</td>
<td>139.1 ± 18.5</td>
</tr>
<tr>
<td>%FMD</td>
<td>4.3 ± 3.2</td>
<td>4.5 ± 4.0</td>
</tr>
<tr>
<td>Excluding medication (n = 138)(^5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>74.1 ± 12.1</td>
<td>72.2 ± 11.9</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>139.9 ± 18.5</td>
<td>136.0 ± 17.5</td>
</tr>
<tr>
<td>%FMD</td>
<td>4.6 ± 3.5</td>
<td>4.2 ± 3.5</td>
</tr>
</tbody>
</table>

\(^1\) FMD, flow-mediated dilation.
\(^2\) Repeated-measures ANOVA for time × equol-status interaction.
\(^3\) n = 87 and 88 for the placebo and soy groups, respectively.
\(^4\) n = 78 and 75 for the placebo and soy groups, respectively.
\(^5\) n = 63 and 75 for the placebo and soy groups, respectively.
TABLE 4
Effects of a soy supplement on vascular function in the equol producers and the nonproducers in the soy group

<table>
<thead>
<tr>
<th></th>
<th>Equol nonproducers (n = 63)</th>
<th>Equol producers (n = 25)</th>
<th>Difference in change (95% CI)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Final visit</td>
<td>Baseline</td>
<td>Final visit</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>74.5 ± 10.7</td>
<td>75.4 ± 15.2</td>
<td>73.2 ± 10.2</td>
<td>72.5 ± 10.0</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>138.2 ± 17.2</td>
<td>140.8 ± 19.3</td>
<td>138.1 ± 15.5</td>
<td>135.1 ± 15.4</td>
</tr>
<tr>
<td>%FMD</td>
<td>4.9 ± 5.5</td>
<td>4.8 ± 5.6</td>
<td>4.8 ± 3.7</td>
<td>5.9 ± 5.2</td>
</tr>
</tbody>
</table>

¹ FMD, flow-mediated dilation.  
² Repeated-measures ANOVA for time × equol-status interaction.  
³ ± SD (all such values).

DISCUSSION

We did not observe a beneficial effect of a soy-protein supplement containing large amounts of isoflavones on vascular function, although effects limited to women capable of equol production cannot entirely be excluded.

To fully appreciate these results, some issues need to be addressed. The strengths of this study were the large sample size and the double-blind randomized design. The number of dropouts did not exceed 25%, as prespecified in the power calculation; thus, the blinding was effective. Although randomization resulted in reasonably well-balanced groups, there were some differences between the soy and the placebo groups at baseline that could have influenced the results. We included baseline systolic and diastolic blood pressure as covariates in the analyses to adjust for these baseline differences, but this did not change the results materially. The number of women who used blood pressure-lowering medication was higher in the placebo group. In Table 3 we present results after all women taking blood pressure-lowering medication (n = 37) were excluded, but this resulted in only small changes in the estimates. The same held true for the subgroup analyses for smoking (data not shown). Although imbalances in the groups, despite randomization, may have slightly influenced the precise estimates, we believe that this did not affect the validity of the overall results of this study.

The lack of an effect may have resulted because of noncompliance. Therefore, we assessed genistein concentrations in the blood sample taken at the final visit, and concentrations were markedly different between the soy and the placebo groups, which demonstrated that compliance was good.

The endothelium—the luminal layer of cells in the vascular wall—plays a major role in vascular function (26). Endothelial cells synthesize nitric oxide (NO) from L-arginine and molecular oxygen through the catalytic activity of endothelial nitric oxide synthase (eNOS). After its synthesis, NO is released, not stored.

Table 3 we present results after all women taking blood pressure as covariates in the analyses to adjust for these baseline differences, but this did not change the results materially. Therefore, we assessed genistein concentrations in the blood sample taken at the final visit, and concentrations were markedly different between the soy and the placebo groups, which demonstrated that compliance was good.

The endothelium—the luminal layer of cells in the vascular wall—plays a major role in vascular function (26). Endothelial cells synthesize nitric oxide (NO) from L-arginine and molecular oxygen through the catalytic activity of endothelial nitric oxide synthase (eNOS). After its synthesis, NO is released, not stored.

In our study in humans we could not confirm such a cardioprotective effect of a soy protein supplement containing isoflavones. For blood pressure, some data from human research are available. Teede et al (10) reported a significant reducing effect on blood pressure of a soy-protein isolate (118 mg isoflavones) compared with casein after 3 mo in postmenopausal women (n = 105) and men (n = 108) in a double-blind randomized trial. The difference in change during the intervention was −3.9 mm Hg for systolic blood pressure and −2.4 mm Hg for diastolic blood pressure, whereas in our trial the results were almost the reverse, 4.3 mm Hg for systolic and 2.0 for diastolic blood pressure. The main difference between our trial and Teede et al’s trial was the duration of the intervention (3 mo in the present trial compared with 12 mo) and the mean age of the participants (an average of 60 y in the present trial compared with an average of 67 y). The active and the placebo interventions (soy-protein isolate and casein) in their trial were similar to the interventions in our trial. The conflicting results could have resulted from a chance finding in either trial. In a study of 25 men and 15 women with hypertension conducted by Rivas et al (39), the effects of a 3-mo intervention with soy milk were compared with those of cow milk; both systolic and diastolic blood pressure decreased after the soy intervention. However, several other trials showed no changes in blood pressure with soy protein (6, 40) or isoflavone tablets (5, 9, 41). Further studies need to be conducted before the true relation between soy protein or isoflavones and blood pressure will be revealed.

For endothelial function, the same inconclusive results as for blood pressure are reported in the literature. The assessment of endothelial function on the basis of FMD is a complex task. Many physiologic factors influence endothelial function, which causes large variability (42). We found no statistically significant effect of soy protein on endothelial function. Teede et al (10) also investigated the effects of soy protein on endothelial function and found no effects in postmenopausal women, whereas endothelial function deteriorated in men. A tablet of 54 mg genistein/d for 6 mo and 1 y, respectively, was reported to improve endothelial function in postmenopausal women (5, 7). Our supplement contained 52 mg genistein/d. In a small 4-wk crossover study (n = 18) with soy-protein isolate, endothelial function also improved;
however, in 3 studies in postmenopausal women ranging from 2 to 8 wk in duration (n = 20–29), no effects were seen (8, 9, 11). Our results are compatible with no effect or, at best, a very limited effect of soy protein on endothelial function.

Recently, the role of the metabolite equol produced from the precursor daidzein has received much attention (23). The intestinal flora produces this relatively strong estrogen agonist in only \( \approx 30\% \) of people after exposure to daidzein. In rodents this metabolite is formed in 100% of animals. This could, in part, be an explanation for the large discrepancy between human and animal research and, therefore, it was hypothesized that only the producers of this metabolite would benefit from interventions with soy protein. We assessed equol-producer status within the intervention group. We could not assess this in the control group because they were not exposed to soy. In the subgroup analyses we found a decrease in systolic and diastolic blood pressure and an improvement in endothelial function in the equol producers and an increase in systolic and diastolic blood pressure and deterioration in endothelial function in the nonproducers of equol.

Because of the small numbers, these differences were not statistically significant; however, the findings do support the hypothesis that only a limited group of women might benefit from soy intervention.

In conclusion, the results of this large, double-blind, 12-mo randomized trial do not support the hypothesis that soy protein containing isoflavones has beneficial effects on vascular function in older postmenopausal women. Whether certain subgroups of women (eg, equol producers) do benefit from soy intervention remains to be elucidated.

SK-K was responsible for the data collection and analysis and the draft of the manuscript. LK was responsible for the data collection and analysis and the critical revision of the manuscript. MLB was responsible for the quality of the vascular measurements and the critical revision of the manuscript. JWL was responsible for the genistein and equol assessments and the critical revision of the manuscript. YTvdS was responsible for the conception and design of the study, for obtaining funding, for the supervision of the data collection and analysis, and for the critical revision of the manuscript. There were no conflicts of interest to disclose. The Solae Company neither controlled nor influenced the contents of the research of this paper nor played any part in the decision to submit this manuscript for publication.

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27. Radomski MW, Palmer RM, Moncada S. Endogenous nitric oxide in-
Dear Sir:

Zimmermann et al (1) showed, in 6–12-y-old Swiss children, that the 95th BMI percentile of the Centers for Disease Control and Prevention (CDC) cutoffs have a higher sensitivity than do the International Obesity Task Force (IOTF) obesity cutoffs to detect obesity based on skinfold thicknesses. They concluded that the CDC cutoffs are superior to the IOTF cutoffs, but this conclusion is misleading for 2 reasons.

It is true that sensitivity is higher with the CDC cutoffs; however, Zimmermann et al did not highlight the higher specificity of the IOTF cutoffs. In Table 5 of Zimmerman et al’s article, it shows that the specificities for detecting obesity with the CDC and the IOTF cutoffs are 97.3 and 98.6, respectively, in girls, and 96.9 and 99.5, respectively, in boys. So, the false-positive rate is twice as high in girls and >6 times as high in boys with the CDC cutoffs.

This tradeoff between sensitivity and specificity is well-known, and focusing on one while neglecting the other is not the best way to compare cutoffs. The reason why the IOTF obesity cutoffs have lower sensitivity (and higher specificity) is simply because they are more extreme, as the authors point out, which leads to lower prevalence rates of obesity (2, 3).

The second concern is that the gold standard used by Zimmermann et al, percentage body fat based on skinfold thicknesses, is set at the 95th percentile of the distribution, which matches the corresponding CDC cutoffs but is lower than the IOTF cutoffs. If the definition of obesity had been based on the 99th percentile for percentage body fat, the comparative results for sensitivity and specificity would have been different, i.e., the sensitivity of the IOTF cutoff would have been much higher. In this instance, as in others, it is important to compare like with like.

Given the continuing rise in the prevalence of child obesity, there is also some benefit in having a more extreme cutoff available to focus on the extreme group of children most at risk of obesity-related complications, such as those with type 2 diabetes (4).

None of the authors had a conflict of interest related to the letter.

Tim J Cole

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United Kingdom
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REFERENCES

Reply to TJ Cole et al

Dear Sir:

We agree with Cole et al that the tradeoff between sensitivity and specificity makes establishing cutoffs for the detection of obesity subjective, to some extent. However, we stand by our conclusion that the obesity cutoff of the Centers for Disease Control and Prevention (CDC) is superior to that of the International Obesity Task Force (IOTF) in Swiss children (1). Although the IOTF cutoff shows slightly higher specificity and has a lower rate of false-positive results, the false-positive rate in our sample was already low with the CDC cutoff, and we would weight the 20–30 point gain in sensitivity with the CDC cutoff as more important.

Cole et al recommend the use of a more extreme cutoff, the 99th percentile of percentage body fat from skinfold thicknesses, as the gold standard for classifying obesity rather than the 95th percentile of skinfold thickness, as used in our study (1). Although this would have improved the comparative results for the IOTF reference, we believe that a child above the 95th percentile for percentage body fat should be identified as obese, because even this degree of adiposity is associated with a high risk for the metabolic syndrome and biomarkers of adverse cardiovascular outcomes (2).

The author had no conflict of interest to disclose.

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REFERENCES

Why is there disinterest in the appreciation of beneficial dietary recommendations?

Dear Sir:

The editorial by Jenkins et al (1), “Too much sugar, too much carbohydrate, or just too much?,” comments on the study by Gross et al (2), in which the adverse effects of the high consumption of refined-carbohydrate foods on the incidence of diabetes were examined. Jenkins et al considered that the seriousness of the present situation should evoke “a wake-up call for radical lifestyle reassessment.” The crucial question concerning this issue is who is listening, let alone acting, with regard to the application of recommended health-improvement measures? A recent editorial in the Lancet, “The catastrophic failures of public health,” stressed that “people are getting fatter and less physically active, and are therefore more prone to killer chronic illnesses such as cardiovascular disease, stroke, cancer, and diabetes” (3).

Understandably, attempts to secure health improvements should begin with the young. In this respect, and revealing the magnitude of the task, a recent School Nutrition Dietary Assessment Study in the United States examined the prevailing desirable dietary recommendations, which includes advice to reduce the average meat serving from 2 to 1.5 ounces (≈43 g); to eliminate milk-based desserts and high-fat meats, cheeses, nuts, nut butters, and desserts; and to reduce sharply the amount of fat added during food preparation. Disappointingly, it was found that only 1% of school lunches complied with these recommendations (4).

It is almost inevitable that changes in epidemiologic ill-health situations will continue. However, it must be appreciated that there will always be subpopulations who are more favored healthwise because of their particular daily habits and practices. For example, it is reported that vegetarians have a lower mortality rate from ischemic heart disease, cerebral vascular disease, and all causes combined because of their particular daily habits and practices. For example, it is reported that vegetarians have a lower mortality rate from heart disease, cerebral vascular disease, and all causes combined because of their particular daily habits and practices. For example, it is reported that vegetarians have a lower mortality rate from heart disease, cerebral vascular disease, and all causes combined because of their particular daily habits and practices.

Major epidemiologic changes of this nature are also occurring in developing populations. In recent decades, urban blacks have experienced slow but steady adverse changes in their patterns of non-communicable diseases concomitant with elevations in socioeconomic status. In South Africa, it was recently reported that the prevalence of diabetes has reached a level far higher in the black than in the white population, namely 5.2% compared with 2.3% (6). Obesity is particularly high among urban black women in South Africa; 44% of this population in Cape Town is obese (7). As for the situation in other developing populations, who are becoming increasingly more Westernized, it is noteworthy that changes in diet, coupled with increasingly inactive lifestyles, have led to major epidemics of obesity in several Asian countries (8). According to the World Health Organization, cardiovascular disease will be the most common cause of death in developing populations by the year 2020 (9).

On the one hand, the health situations and outlooks described in both developed and urban developing populations are consistent with rising life expectancies. For example, women in Japan—followed closely by their French and Swiss peers—now have a 1 in 20 chance of living to the age of 100 y (10). Yet, on the other hand, far fewer improvements have been occurring in “healthy life expectancies” (11).

Neither author had a conflict of interest to disclose.

Alexander Walker
Betty Walker

REFERENCES

Reply to A Walker and B Walker

Dear Sir:

Walker and Walker, in their comments about our editorial accompanying the article by Gross et al (1) on the consumption of refined carbohydrates and disease, note that preventive strategies should begin with the young and that developing nations are far from free of...
the problems of overconsumption, especially affluent populations in urban centers. They also mention that certain subgroups may be more favored health-wise because of their particular daily habits and practices. Vegetarians are singled out as an example of a group who have age-adjusted lower rates of coronary heart disease, stroke, and all-cause mortality (2). This example is relevant at a time when Gary Fraser and friends have published their assessment of the effect of vegetarian diets on chronic disease and longevity (3). They concluded that a Californian Seventh-Day Adventist vegetarian lifestyle including diet, exercise pattern, and tobacco and alcohol use results in a marked increase in longevity. For men aged >30 y, life expectancy was 7.28 y longer than for the general population; for women, life expectancy was 4.42 y longer (4). A more modest effect was found in an Oxford study of British vegetarians, which also showed a greater life expectancy in vegetarians than in nonvegetarians. Vegetarians, and certainly vegans (5), tend to weigh less than their omnivorous counterparts, and their incidence of diabetes has been reported to be lower. Are vegetarians spared because they consume a greater amount of unrefined traditional carbohydrate foods, which have a lower glycemic index, or do they simply eat fewer calories than they expend? Certainly, the most recent analysis of the Nurses’ Health Study again showed a protective effect of both cereal fiber and a low glycemic index in reducing the risk of diabetes after adjustment for body weight and exercise (6).

Walker and Walker mention that diabetes is on the rise in urban and suburban Africa. Lack of exercise is noted as one reason for this change, but the shift to a more Western dietary pattern, which is characterized by foods with higher glycemic indexes, is another reason.

Unfortunately, without more research and education globally, these changes in human health are inevitable because it appears that we have been given what we have asked for: an abundant supply of inexpensive food and a lifestyle that requires minimal energy expenditure. The industrial sector has simply done what we have paid them to do. Since when has money been made on food that is “good for you” but that does not “taste good”? What automobile or kitchen appliance was ever sold because “you have to expend more energy to use this model than the previous one”? Taste, ease and speed of food preparation, and labor-saving devices of all kinds are the order of the day. We should not expect citizens of less industrialized nations to have goals different from our own. In fact, human migration patterns indicate a move from countryside to cities and from less to more industrialized societies.

Perhaps we should reflect on the lives of many modern-day humans, who spend much of their time hunched over word processors, who tend to eat high-calorie comfort foods and foods with high glycemic indexes, and expend little time on exercise. Are we setting a good example to the developing world? Is eating less-processed foods and working out at the gym the whole answer, or is there more to life (and nutrition)?

DJAJ has served as a consultant to Solale (St Louis) and to the Almond Board of California for the past 5 y, has received honoraria from Oldways Preservation Trust (Boston) and the Almond Board of California, and is on the Scientific Advisory Board of Loblaws Supermarket, Sanitarium Company (Sydney, Australia), Herbalife International (Century City, CA), Nutritional Fundamentals for Health (Montreal), Pacific Health Laboratories Inc (Matawan, NJ), and the California Strawberry Commission. CWCK has received honoraria from the Almond Board of California.

David JA Jenkins
Cyril WC Kendall
Augustine Marchie
Livia SA Augustin

REFERENCES
Erratum


In Table 1 (page 127), the baseline values of 25(OH) vitamin D in the normal calcium group should be 84.1 ± 19.9 nmol/L for weight maintenance and 73.3 ± 21.9 nmol/L for weight loss, and those in the high calcium group should be 68.9 ± 14.6 nmol/L for weight maintenance and 61.6 ± 22.0 nmol/L for weight loss.

Because the values change consistently across the categories, the statistics and interpretation of the data remain unchanged.

Erratum


On page 1179, the legend for Figure 1 should read as follows:

**FIGURE 1.** Mean (±SE) change in minimal coronary artery diameter according to intake of different nutrients, with adjustments as in Table 2 (see footnote 1), except that total fat was not adjusted for carbohydrate, and carbohydrate and protein were also adjusted for polyunsaturated fat. These models estimate the effect of saturated fat replacing other fats (monounsaturated or polyunsaturated), monounsaturated fat replacing other fats (saturated or polyunsaturated), polyunsaturated fat replacing other fats (saturated or monounsaturated), total fat replacing carbohydrate, carbohydrate replacing saturated or monounsaturated fat, and protein replacing saturated or monounsaturated fat. Median intakes (% of energy) for quartiles 1–4 were as follows: saturated fat (6.1, 7.8, 9.5, and 12.0), monounsaturated fat (6.9, 8.6, 10.7, and 13.0), polyunsaturated fat (3.9, 5.2, 6.1, and 7.5), total fat (17.6, 21.7, 27.0, and 31.9), carbohydrate (47.1, 55.6, 60.5, and 68.9), and protein (12.7, 15.8, 18.0, and 21.2). P for trend = 0.001 (saturated fat), 0.40 (monounsaturated fat), 0.04 (polyunsaturated fat), 0.48 (total fat), 0.20 (protein), and 0.001 (carbohydrate).

Understanding the relevance of current knowledge on drug-nutrient interactions is a major challenge to the health care community. Dependence on the use of pharmacologically active substances—including prescription medications, over-the-counter pharmaceuticals, and dietary supplements—to restore or preserve health or to manage medical disorders has increased in the past several decades. Large segments of the American population, especially the elderly, consume one or more prescription medications daily. For many persons, long-term survival would be at risk without these medications. Yet, clinicians rarely advise their patients about what to eat and what not to eat while taking these prescribed medications. There has been a major emphasis on the application of current knowledge about drug-drug interactions; however, except for a few notable drug-nutrient interactions (e.g., with grapefruit juice, alcohol, and caffeine), few health care practitioners are aware of or advise their patients on how to minimize the risks associated with drug-nutrient interactions. In the Handbook of Drug-Nutrient Interactions, the authors provide a review of the scientific literature on known drug-nutrient interactions and offer an assessment of their relative effects on the patient’s well being.

This book is organized on the premise that a classification system based on location and mechanism of interaction would help to design management strategies. Six main sections are subdivided into 26 chapters, all of which are authored by respected authorities in their fields. Four sections tended to follow the abovementioned classification system and are entitled “Overview of Drug-Nutrient Interactions,” “Influence of Nutritional Status on Drug Disposition and Effect,” “Influence of Food or Nutrients on Drug Disposition and Effect,” and “Influence of Pharmaceuticals on Nutrition Status.” The chapters included in these sections are well documented and provide a review of the known sites of interaction and the enzymes and transport molecules involved. The authors of several chapters make excellent use of tables and figures to present data on the interactions between drugs and substances in foods, the effect of nutritional status on the response to drug therapy, and the effect of drug therapy on nutritional status. Two chapters summarize science-based information on the interaction of dietary supplements with drugs and nutrients. Where specific data are lacking, the authors provide discussions on the likely mechanisms involved based on knowledge of biochemical and physiologic processes and cite the specific need for further research. Scientists engaged in many areas of clinical research will find the comprehensive reviews to be an excellent background for planning and designing studies.

The last 2 sections of the book are devoted to drug-nutrient interactions as they relate to specific segments of the population.

In Section 5, the individual chapters are devoted to the drug-nutrient interactions that have special importance to certain life stages, including infancy and childhood, pregnancy and lactation, and the elderly. In Section 6, the chapters focus on drug-nutrient interactions relating to long-term medical conditions. Topics in this section include cancer, organ transplantation, immune-related diseases, chronic infections, long-term antimicrobial use, and long-term enteral nutrition. Extensive tables that summarize clinical recommendations enhance the science-based information in these 2 sections. The clinical orientation of the book provides an ideal resource for health care practitioners.

This book will have additional value for persons who anticipate its frequent use as a reference source because it comes with a CD-ROM that contains an Adobe eBook/PDA version. This allows the book to be viewed on one computer and one hand-held device. With the use of this technology, a rapid search for a specific topic can be made. In summary, this book is written to assist health professionals in using and providing information on the effects of drug-nutrient interactions.

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Metabolic and Therapeutic Aspects of Amino Acids in Clinical Nutrition, second edition, is a compilation of 44 chapters written by 81 authors as an update to the first edition, which was published in 1995. This edition is dedicated to the memory of Bernard Beaufre and Peter Reeds, both of whom were recent contributors to the field of amino acid nutrition in health and disease. The book begins with a general introduction by John M Kinney to the development and scientific progression of amino acid nutrition and metabolism in the 20th century. This book is written for an audience that may be looking for a sound presentation on amino acid metabolism as it applies to health and disease in humans, and the reader should appreciate the scientific presentation and documentation in each chapter. The first section of the book describes the fundamentals for assessing amino acid nutriture: it explains proper sample preparation and storage and the analysis of amino acids by using ion exchange chromatography or HPLC; shows the validity of various methods; and includes some normal
values in human plasma, muscle, and red blood cells. For the benefit of readers who might not be familiar with flux measurements obtained with the use of stable isotopes, a chapter is included that explains this approach in the study of amino acid metabolism.

Topics that are thoroughly covered are the cellular transport of amino acids, the role of amino acids in gluconeogenesis and ketogenesis, and the importance of the urea cycle and its regulation. Much detail is provided in various chapters on the role in nutrition and metabolism of amino acids, such as glutamate, glutamine, alanine, and the branched-chain amino acids, which have been studied in great detail in recent years, and the text includes valuable information on the role of arginine in providing regulation of both the urea cycle and the production of nitric oxide. Updated information is also presented on amino acid signaling of hormonal responses, especially the response of insulin, and on the role of amino acids, especially leucine, in protein synthesis and degradation.

This information is amplified in several chapters that cover the possible supportive role of amino acid nutrition in patients with various diseases such as cancer, diabetes, and gastrointestinal diseases, as well as the possible role of certain amino acids during exercise. A thorough discussion of amino acid requirements in humans, from healthy neonates to the elderly, is presented along with information on how these requirements vary with different diseases. All of the amino acids are covered in the requirement section, whereas the detailed metabolism of most of the essential amino acids (eg, trp, lys, thr, his, met-cys, and phe-tyr), because they have not been studied lately in relation to human nutrition, does not receive extensive coverage. Because this book focuses on amino acid nutrition and metabolism as they relate to human nutrition, some important areas that have not yet been related to human nutrition, such as the question of ileal or whole gastrointestinal tract digestibility (ie, amino acid bioavailability), are not covered. Despite this, this volume provides information about the difference in digestibility (ie, amino acid bioavailability) to human studies. For example, the conclusion by Gabriel Fernandes that “...there is strong support for the contention that dietary fish oil can decrease OVX [ovariectomy]-induced bone loss” indicates a possible mechanism, but Fernandes added that “...additional studies are needed to determine the effects of...n-3 fatty acids... in protecting against perimenopausal bone loss....” Will this enable the experienced clinical dietitian to make specific recommendations to the perimenopausal woman? On the other hand, would she not have known what Susan Barr concluded, perfectly sensibly, ie, “...it would be prudent for committed vegans to choose fortified foods and/or supplements to ensure they can meet current recommendations for these nutrients.”

Many chapters contain extensive and useful summaries. For example, the chapter by Douglas Kiel contains long summaries of the many publications that have dealt with the effects of smoking and alcohol on bone health. This chapter, like many others, contains many references so that the reader interested in a particular subject can gain a good introduction to what is known or needs to be known. Other chapters deal with topics that one might not easily find summarized elsewhere, eg, chapters on fluoride, lead, phytoestrogens, or bone health in cystic fibrosis. The book, therefore, can be a valuable reference and should be available in general and departmental libraries.

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With Appreciation

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Polyphenols: antioxidants and beyond1–3

Augustin Scalbert, Ian T Johnson, and Mike Saltmarsh

ABSTRACT
Research on the effects of dietary polyphenols on human health has developed considerably in the past 10 y. It strongly supports a role for polyphenols in the prevention of degenerative diseases, particularly cardiovascular diseases and cancers. The antioxidant properties of polyphenols have been widely studied, but it has become clear that the mechanisms of action of polyphenols go beyond the modulation of oxidative stress. This supplemental issue of The American Journal of Clinical Nutrition, published on the occasion of the 1st International Conference on Polyphenols and Health, offers an overview of the experimental, clinical, and epidemiologic evidence of the effects of polyphenols on health. Am J Clin Nutr 2005; 81(suppl):215S–7S.

KEY WORDS Polyphenols, flavonoids, antioxidants, health, cardiovascular diseases, cancers

Polyphenols are the most abundant antioxidants in the diet. Their total dietary intake could be as high as 1 g/d, which is much higher than that of all other classes of phytochemicals and known dietary antioxidants. For perspective, this is ~10 times higher than the intake of vitamin C and 100 times higher that the intakes of vitamin E and carotenoids (1, 2). Their main dietary sources are fruits and plant-derived beverages such as fruit juices, tea, coffee, and red wine. Vegetables, cereals, chocolate, and dry legumes also contribute to the total polyphenol intake.

Despite their wide distribution in plants, the health effects of dietary polyphenols have come to the attention of nutritionists only rather recently. Until the mid-1990s, the most widely studied antioxidants were antioxidant vitamins, carotenoids, and minerals. Research on flavonoids and other polyphenols, their antioxidant properties, and their effects in disease prevention truly began after 1995 (Figure 1). Flavonoids were hardly mentioned in textbooks on antioxidants published before that date (3). The main factor that has delayed research on polyphenols is the considerable diversity and complexity of their chemical structures.

Current evidence strongly supports a contribution of polyphenols to the prevention of cardiovascular diseases, cancers, and osteoporosis and suggests a role in the prevention of neurodegenerative diseases and diabetes mellitus (4). However, our knowledge still appears too limited for formulation of recommendations for the general population or for particular populations at risk of specific diseases. Evidence for a reduction of disease risk by flavonoids was considered “possible” for cardiovascular diseases and “insufficient” for cancers in a recent report from the World Health Organization (5). The objectives of the 1st International Conference on Polyphenols and Health (Vichy, France, November 18–21, 2004) were to offer an overview of our current knowledge on the associations between polyphenol intake and disease and health and to discuss key issues awaiting resolution. More than 350 communications from >30 countries were presented. The articles included in this volume correspond to the invited lectures presented at the conference.

Much of the evidence on the prevention of diseases by polyphenols is derived from in vitro or animal experiments, which are often performed with doses much higher than those to which humans are exposed through the diet. One purpose of the conference and of this volume was to review some of the evidence for health effects of polyphenols in humans, from both clinical trials and epidemiologic studies. Polyphenols clearly improve the status of different oxidative stress biomarkers (6). Much uncertainty persists, however, regarding both the relevance of these biomarkers as predictors of disease risk and the appropriateness of the different methods used (7). Significant progress has been made in the field of cardiovascular diseases, and today it is well established that some polyphenols, administered as supplements or with food, do improve health status, as indicated by several biomarkers closely associated with cardiovascular risk (8–10). Epidemiologic studies tend to confirm the protective effects of polyphenol consumption against cardiovascular diseases (11). In contrast, evidence for protective effects of polyphenols against cancers, neurodegenerative diseases, and brain function deterioration is still largely derived from animal experiments and in vitro studies (12, 13); we await the discovery of predictive biomarkers for such diseases or large intervention studies, similar to those performed with nonphenolic antioxidants (14).

One of the major difficulties of elucidating the health effects of polyphenols is the large number of phenolic compounds found in food (15), yielding differing biological activities, as shown in several in vitro studies (16, 17). Major differences in bioavailability are now well established, and the influence of structural factors is better understood (18). This issue was discussed at length during the conference. The active compounds may not be

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the native polyphenols found in food, which are most often tested in in vitro studies; they are more likely to be metabolites (19). The importance of microbial metabolites has been emphasized in some recent studies, as exemplified by equol, the major metabolite of daidzein (20). Polyphenols are extensively conjugated in the body, and nonconjugated metabolites most often account for a minor fraction of the circulating metabolites. Very little is currently known regarding the biological activities of these conjugated metabolites (1). Glucuronides of isoflavones and epicatechin were shown to have much weaker estrogenic activity and provided no protection against oxidative stress in cells grown in vitro (21, 22). These findings suggest that many of the in vitro studies published to date must be reevaluated, in light of the new data on polyphenol bioavailability.

A considerable body of literature supports a role for oxidative stress in the pathogenesis of age-related human diseases and a contribution of dietary polyphenols to their prevention. The complex relationships between antioxidant status and disease are still poorly understood and have been studied intensively. For many years, polyphenols and other antioxidants were thought to protect cell constituents against oxidative damage through scavenging of free radicals. However, this concept now appears to be an oversimplified view of their mode of action (23). More likely, cells respond to polyphenols mainly through direct interactions with receptors or enzymes involved in signal transduction, which may result in modification of the redox status of the cell (24–26). Both antioxidant and prooxidant effects of polyphenols have been described, with contrasting effects on cell physiologic processes. As antioxidants, polyphenols may improve cell survival; as prooxidants, they may induce apoptosis and prevent tumor growth (12). However, the biological effects of polyphenols may extend well beyond the modulation of oxidative stress. One of the best-known examples involves the interaction of soy isoflavones with estrogen receptors and the effects of these compounds on endocrine function. These effects could explain the prevention by isoflavones of bone resorption among postmenopausal women (27). A detailed understanding of the molecular events underlying these various biological effects is essential for evaluation of the overall impact on disease risk and progression.

The current evidence for protective effects of polyphenols against diseases has generated new expectations for improvements in health, with great interest from the food and nutritional supplement industry regarding promotion and development of polyphenol-rich products. However, it is still impossible to evaluate the individual and societal benefits that increases in polyphenol intake could have for the general population or for particular groups at specific disease risk. Furthermore, a significant increase in the consumption of polyphenols, as for many other phytomolecules, may not be without risks (28). Some hazards associated with the consumption of polyphenols are documented, but evaluation among humans is still very limited. Lastly, we should not forget that many polyphenols have a taste and/or a color (29); food must be not only good for health but also acceptable to consumers.

Integration of the results of past and future experiments in various disciplines, including biochemistry, cell biology, physiology, pathophysiology, epidemiology, and food chemistry, will be needed to identify the most effective polyphenols and to determine the optimal levels of intake for better health. The present research efforts will coordinate with current efforts to identify more accurate biomarkers of risks for nutrition-related diseases and should lead to dietary recommendations and the formulation of new food products contributing to good health.


The history of β-carotene and cancers: from observational to intervention studies. What lessons can be drawn for future research on polyphenols?1–3

Serge Hercberg

ABSTRACT
An important question being raised by nutritionists today is whether available scientific data support an important role for polyphenols in the prevention of pathologic conditions that represent an important public health burden, such as cardiovascular diseases, cancers, and osteoporosis. More broadly, when can we consider scientific knowledge sufficient to allow specific public health implications and recommendations? The history of the relationship between β-carotene and cancer illustrates the complexity of the research process leading to the demonstration of a causal relationship between nutritional factors and the prevention of disease. The β-carotene story, which has developed in the past 30 y, is particularly significant and illustrative because of apparent controversies that are far from resolved. This is an extremely interesting example from which many lessons can be learned. For β-carotene, we need to collect sufficient information from experimental, clinical, and epidemiologic research before we support any specific public health recommendations. The same principles must be applied to recommendations regarding polyphenols (in particular, which polyphenols, at which doses, to achieve which benefits for which populations). If these questions are not answered, then we run the risk of needing to renounce recommendations regarding polyphenols in the future, damaging the credibility of nutritional recommendations for public health. Am J Clin Nutr 2005;81(suppl):218S–22S.

KEY WORDS β-Carotene, cancers, recommendations, intervention trial, polyphenols

INTRODUCTION
Although their existence has been known for many years, the interest in polyphenols has increased sharply in the past 2 decades. This “boom” is reflected by the major increase in the number of scientific articles published in the past 20 y; when performing a MEDLINE search with the keyword polyphenols, we found that 7 articles had been published in 1981, 15 in 1990, 53 in 1995, 125 in 1998, and 437 in 2002. In particular, the number of articles on the potential benefits of polyphenols in preventing chronic diseases has increased. Industry has not been oblivious to this accumulation of scientific data, and the result is that today we can buy “over-the-counter” supplements containing polyphenols and products fortified with polyphenols; indeed, the food industry would like to make claims regarding the supposed health benefits of polyphenols in their products.

Currently, however, the main question is whether available scientific data justify an important role for polyphenols in disease prevention through nutrition, particularly with respect to pathologic conditions with an important public health burden, such as cardiovascular diseases, cancers, and osteoporosis. More generally, when can we consider scientific knowledge sufficient to allow specific public health applications and recommendations? In this respect, the history of the relationship between β-carotene and cancer illustrates the complexity of the research process leading to the demonstration of causal relationships between nutritional factors and disease prevention. This is an extremely interesting example from which many lessons can be learned, as suggested by several authors (1–4).

The β-carotene story that developed in the past 30 y is particularly illustrative of the intricacies of nutritional research because of apparent controversies that are far from resolved. The story has its ups and downs and could be titled “The rise and fall of β-carotene,” “β-Carotene and health: the search for the holy grail,” “Song of the sirens,” “Dr. Jekyll and Mr. Hyde,” or “The never-ending story.”


Background
The story began ~30 y ago, between 1970 and 1980, with the conjunction of mechanistic theories regarding the role of free radicals considered capable of inducing numerous pathologic processes (especially carcinogenesis and atherosclerosis) and the interest in nutrients such as β-carotene that, because of their antioxidant capacity, could prevent such harmful effects. Evidence from experiments with animals indicating that β-carotene could have anticancer activity began to accumulate, and data from observational epidemiologic studies of cancer and vegetable, fruit, and β-carotene intake became available.

A catalyst was necessary to make sense of all of these elements and to provoke a major interest in β-carotene. This came in the...

The role of free radicals in carcinogenesis

In the 1970s, the indispensable but potentially dangerous role of oxygen had been known for more than a century (6). Jules Verne, the French writer and visionary, wrote in 1865, in his novel “From the earth to the moon,” that “oxygen... this gas without a smell, eminently vital, can cause the most serious disorders in the organism.”

Oxygen toxicity in laboratory animals was first described in 1878 (7) and was established in 1899 (8). The first experiments investigating a free radical reaction were reported in 1894 by Fenton (9). However, it was not until the late 1940s to early 1950s that retrolental fibroplasia among premature newborns was recognized as being attributable to oxygen toxicity (10), and it was not until the late 1960s to early 1970s that newborn bronchopulmonary dysplasia and adult respiratory distress syndrome were appreciated by the medical community (11). Moreover, the presence of free radicals in biologic systems was not generally considered likely until the discovery of superoxide dismutase in 1969 (12), although the basis of oxygen toxicity and X-irradiation was proposed to be a common free radical mechanism and the radical theory of aging was hypothesized in the 1950s (13).

It was at a meeting of the World Health Organization in the mid-1960s that a Russian toxicologist, Professor Sanojki, referred to various degenerative diseases as being “rusting diseases” and linked their cause to free radicals (4). Several studies performed between 1972 and 1980 suggested that free radical production directly and indirectly played a major role in carcinogenesis.

Animal studies supporting the anticancer activity of β-carotene

After the first reports of antitumor effects of vitamin A in animal models, which appeared in the 1960s, investigators were curious to know whether β-carotene, the precursor of vitamin A, had antitumor effects. In 1973, Dorogokupla (14) showed that subcutaneous tumors induced with injection and topical application of dimethylbenzanthracene developed at a slower rate among rodents fed a diet supplemented with “unlimited amounts of red carrots,” compared with animals fed the unsupplemented diet. In 1977, Epstein (15) reported that tumors induced with ultraviolet radiation appeared at a slower rate among hairless mice given injections of a β-carotene solution, compared with mice given injections of a placebo solution. More and more experiments suggested that carotenoids, irrespective of their vitamin A activity, were able to prevent or slow the growth of tumors induced with ultraviolet light or dimethylbenzanthracene. From a mechanistic viewpoint, in vitro and in vivo studies showed that carotenoid pigments were able to scavenge highly reactive species such as oxygen and other free radicals and thereby prevent harmful effects of these species (16).

Epidemiologic data on β-carotene intake and the risk of cancer

In the early 1980s, there was already substantial epidemiologic evidence indicating associations between high fruit and vegetable consumption, high estimated β-carotene intake in the diet and/or high blood concentrations of β-carotene, and lower incidences of cancers, particularly lung cancer (16). The first epidemiologic studies investigated the relationship between β-carotene and cancer almost by accident, because dietary questionnaires designed for more general purposes happened to ask about the main local β-carotene source and it was realized later that consumers of those particular foods had lower cancer risks.

Five prospective and 15 retrospective case-control, questionnaire-based studies of populations in 8 different countries were published at the end of the 1970s (5). A relative risk of cancer (lung, stomach, colorectal, or other) of 1.5–2.0 was typically observed in comparisons of groups with low and high quartiles or quintiles of β-carotene intake, as estimated with dietary questionnaires.

Using data from experimental studies, animal studies, and observational epidemiologic studies, Peto et al (5) highlighted in 1981 the potential public health significance of β-carotene and the need for future controlled trials. In their article, Peto et al (5) concluded, “it is most unlikely that this inverse cancer risk association will disappear entirely with future observational studies, but the inverse association may be an artifact, due merely to association of β-carotene ingestion with some truly protective dietary habit(s) or component(s) or avoidance of some truly harmful habits or components.” Randomized, population-based trials are direct tests of hypothesis-generating consistent associations noted in observational studies.


During the 1980s, much experimental evidence accumulated in favor of cancer-preventing effects of dietary β-carotene and of β-carotene administered through injection or topical application. In National Cancer Institute preclinical studies, β-carotene and other carotenoids inhibited tumorigenesis in head/neck, mammary, colon, bladder, and skin cancer models in vivo and in mammary organ cultures in vitro.

During this period, overwhelming observational evidence provided support for the association between greater consumption of carotenoid-rich foods (specifically, higher β-carotene intake) and lower cancer risk. Finally, >125 case-control or cohort studies relevant to the association between β-carotene (assessed with dietary intake or biochemical measurements) and cancer were conducted between 1980 and 1990, with various measures in diverse populations. Most of the studies consistently showed protective effects (1). Relative risk increases of 50–150% (ie, relative risks of 1.5–2.5) were typically reported for the lowest vegetable/fruit or β-carotene intake categories, compared with the highest. These observed associations were relatively strong and occurred in studies of men and women (17), different racial groups (18), and current smokers, former smokers, and nonsmokers (19) and therefore appeared to be quite generalized (1). These results could have substantial potential public health implications. Taken together, at the end of the 1980s, the investigation of carotene-rich vegetables, β-carotene intake, and serum or plasma β-carotene concentrations in relation to cancer (especially lung cancer) provided the most persuasive evidence available in the diet-cancer epidemiologic literature for a protective association, in terms of both magnitude and consistency (1, 3).

At the beginning of the 1990s, only data from observational research and experimental work were available for formulation of nutritional recommendations regarding β-carotene intake. However, on the basis of the criteria of causality, such as 1) consistency, 2) strength of the association, 3) dose-response gradient, and 4) biologic plausibility, the evidence could have been considered sufficient to support additional recommendations and specific claims regarding either carotenoid-rich foods or β-carotene intake in particular. It is certain that the then-available data contributed to the increasing popularity of vitamin supplements during the 1980s.

In the 1990s, many impatiently but confidently awaited the results of intervention trials that had begun in the 1980s. Well-designed, randomized intervention trials are considered to provide highly relevant, specific, convincing evidence of causality between nutrient intake and pathologic conditions (for instance, cancer risk) and thus play an important role in the development of nutrition-related recommendations. Randomized trials avoid most of the biases inherent in observational studies. Randomized intervention trials addressing the role of supplementation with antioxidant micronutrients in cancer prevention were started in the 1980s, and some were completed by the mid-1990s. The results of 5 large intervention trials were published between 1993 and 2000.

The Nutritional Intervention Trials in Linxian (20) were developed in a general Chinese population. In that series of studies involving a population of 29 584 subjects (mostly nonsmokers), the use of daily doses of 15 mg β-carotene, 30 mg vitamin E, and 50 μg selenium during a > 5-year period significantly reduced the overall mortality rate by 9%, the rate of death resulting from all cancers by 13%, and the rate of death resulting from gastric cancer by 21%. Because of the nature of the combination micronutrient supplement, intervention effects from this trial could not be attributed with certainty to any one of the 3 nutrients. These results were consistent with those of observational studies of the protective effects of antioxidant nutrients, especially β-carotene.

A few months after the publication of that trial, other trials did not confirm those encouraging results. However, it is important to bear in mind that the Chinese population of the Linxian area is known to have high incidences of gastric and esophageal cancers and high frequencies of nutritional deficiencies.

The Alpha-Tocopherol and Beta-Carotene (ATBC) Cancer Prevention Study began in Finland in 1985 (21). The subjects were 29 133 male heavy smokers, 50–59 y of age, enrolled in a randomized, double-blind trial. Supplementation consisted of 20 mg β-carotene/d or 50 mg α-tocopherol/d, in a 2 × 2 factorial design. The results for β-carotene were surprising. No benefits with respect to the prevention of lung cancer were seen. Instead, a significant 16% increase in the incidence of lung cancer was noted among those receiving the β-carotene supplements, suggesting an adverse effect. This 16% increase (95% CI: 2–33%) in lung cancer incidence was clearly inconsistent with the 2-fold risk reduction attributed to high β-carotene intake in numerous observational studies, and it essentially ruled out a primary preventive effect on lung cancer among smokers subjected to a regimen of daily intake of a 20-mg β-carotene supplement for 5–8 y. The significance of this unexpected finding was increased by the fact that the β-carotene group also experienced an 8% increased overall mortality rate, including an apparent increase in the rate of death attributable to ischemic heart disease.

The interpretation of 2 decades of research on the relationship between β-carotene from vegetables and fruit and lung cancer was suddenly called into question by these findings. Thus began the “fall of β-carotene.”

A few months later, similar results were reported from the Carotene and Retinol Efficacy Trial (CARET). This multicenter trial, which included 18 314 heavy smokers and professionally exposed subjects (22), was terminated 21 mo earlier than planned. After 4 y, increases in the lung cancer incidence and total mortality rate were observed in the group receiving supplementation with 30 mg β-carotene/d and 25 000 IU (13 664 retinol equivalents) retinyl palmitate/d. A total of 388 subjects developed lung cancer, ie, a 28% increase in lung cancer incidence among participants who received the β-carotene/retinyl palmitate combination daily for an average of 4 y, compared with participants who received a placebo.

Until these CARET results were announced and published in early 1996, the ATBC study findings were viewed cautiously and, by some, with skepticism. Thereafter, the concordant data from the ATBC study and the CARET created a striking apparent contradiction with previous results from observational epidemiologic studies.

In contrast to findings from the ATBC study and the CARET, the Physicians’ Health Study (23), which began in 1982 and involved 22 071 US male subjects (primarily nonsmoking physicians), 40–84 y of age, showed no effective difference in lung cancer incidences between the β-carotene group (50 mg on alternate days) and the placebo group after 12 y of supplementation. This was based on only 66 and 71 lung cancer cases in the β-carotene and placebo groups, respectively, and represented a nonsignificant 7% reduction. No adverse or beneficial effects were observed in the 11% of smokers in the β-carotene group. In the Women’s Health Study (24) as well, no effect of vitamin E (600 IU) or β-carotene (50 mg on alternate days) supplementation was observed among 39 876 apparently healthy, female, health care professionals, ≥ 45 y of age, after a treatment duration of 2.1 y and a median total follow-up period of 4.1 y.

The results of trials published in the 1990s did not support an association or role for supplemental β-carotene, at the doses and durations of supplementation tested, in cancer prevention in populations. These results were seemingly at odds with those from observational epidemiologic studies. These trial results make it highly unlikely that pharmacologic doses of supplemental β-carotene are beneficial for prevention of most lung cancers, and they provide strong evidence for adverse effects (eg, increased tumor promotion or progression) among smokers.

These studies raised the issue of interpretation of cohort and case-control studies (eg, is it the β-carotene in the diet?) and reopened the question of the safety of β-carotene supplements, which had long been considered resolved. The results require that caution be exercised in recommending supplemental β-carotene and argue against changing dietary recommendations from foods to nutrient specificity at this time.

After publication of the negative effects of 2 of these trials, there was a total change in the view of the role of β-carotene. Some researchers in the scientific community called for an end to additional chemoprevention studies involving β-carotene and expressed concern about the dangers and liabilities of its unregulated use (3, 25). Results from the ATBC study and the CARET...
led the Food and Drug Administration to disallow health claims for \( \beta \)-carotene related to cancer prevention, under the Dietary Supplement Health and Education Act of 1994 (26), in part because no recognized scientific body (such as the National Institutes of Health or the National Academy of Sciences) had provided “authoritative statements” supporting such claims.

Therefore, the hopes raised in the 1980s concerning the protective effect of \( \beta \)-carotene totally disappeared at the end of the 1990s; it was as if Dr Jekyll became Mr Hyde. \( \beta \)-Carotene not only was considered not to be protective but also was potentially deleterious, with an increased risk of lung cancer among smokers. Nevertheless, the \( \beta \)-carotene experience seems to have had minimal impact on the dietary supplement industry, which has seen its sales double in the past 5 y to > $13 billion from > 20 000 products (27).

**2004: THE NEVER-ENDING STORY**

In this context, a new revival was recently provoked by the presentation of preliminary results of the Supplémentation en Vitamines et Minéraux Antioxydants (SU.VI.MAX) study (28), a randomized, double-blind, placebo-controlled, primary prevention trial among 13 017 middle-aged subjects who received a combination of 120 mg vitamin C, 30 mg vitamin E, 6 mg \( \beta \)-carotene, 100 \( \mu \)g selenium, and 20 mg zinc, compared with placebo. After a mean follow-up period of 7.5 y, 88 men in the intervention group developed cancers, compared with 124 men in the placebo group, which yields a relative risk of 0.69. This difference was reflected in higher incidences of digestive, respiratory, and skin cancers in the placebo group. Among women, no statistically significant difference in the occurrence of cancer was observed between the groups. A total of 174 deaths occurred during the trial, 76 in the intervention group and 98 in the placebo group. No overall significant difference in rates for any cause of death was observed between the groups. Again, a sex-group interaction was observed, showing a significant protective effect (relative risk: 0.63) of antioxidants among men and no effect (relative risk: 1.03) among women.

Therefore, a combination of antioxidants including \( \beta \)-carotene, at doses achievable through the diet, may have protective effects on mortality rates and on the total number of cancers among apparently healthy men, with no evident increase in cancer risk. These doses correspond to those in a healthy diet with a high consumption of fruits and vegetables, confirming the results of prospective observational studies.

Baseline \( \beta \)-carotene and vitamin C status among men in the SU.VI.MAX study was lower than that among women. The ineffectiveness of supplementation among women might have been attributable to their better baseline antioxidant status. The improved antioxidant nutritional status achieved with supplementation among men, which proved protective against cancer, supports the results of other prospective observational studies of the benefits of consuming large amounts of fruits and vegetables. This reinforces the general recommendations of a lifelong diversified diet rich in foods that are good sources of antioxidant nutrients.

The SU.VI.MAX. study has not led to a total “rehabilitation” of \( \beta \)-carotene as a nutrient capable of reducing cancer incidence. The design of the study does not enable us to confirm a specific protective role of \( \beta \)-carotene, but it does raise some new issues. The apparent discrepancies between observational and randomized studies and among the randomized trials could be explained by the choice of study population (general or high-risk subjects, well-nourished or deficient, with adequate or insufficient antioxidant status), the different doses of supplementation (nutritional or higher), the number of antioxidants tested (1, 2, or more), and the type of administration (alone or in balanced association).

The different studies available, and especially the intervention trials, suggest that the effects of \( \beta \)-carotene differ according to the dose and the type of population studied. On the basis of available evidence, it currently seems wise to advocate a diet rich in fruits and vegetables, rather than consumption of specific \( \beta \)-carotene supplements, to decrease cancer risk. Taking into consideration the possible prooxidant role of high doses of \( \beta \)-carotene (29), it is recommended that long-term use of such supplements at high doses be limited, especially among subjects with high risks of cancer, such as smokers.

**WHAT LESSONS FOR POLYPHENOLS?**

Returning to research on polyphenols, we should bear in mind that, compared with \( \beta \)-carotene, research on the health effects of polyphenols started more recently and fewer data are available. This relatively late interest is explained largely by the complexity of their chemical structures, the numerous different phenolic compounds and potentially active metabolites that exist, and the scarcity of intake data.

As was the case for \( \beta \)-carotene, experimental studies with animals and cultured human cell lines tend to support a role for polyphenols in the prevention of cardiovascular diseases, cancers, neurodegenerative diseases, diabetes mellitus, and osteoporosis. We must be cautious, however, because most of those studies were conducted at doses or concentrations that probably do not occur in the human body under normal conditions. Clinical studies have led to contradictory results. Convergent data from epidemiologic studies showed an inverse relationship between the consumption of foods rich in polyphenols and the risk of myocardial infarction or cancer; however, those were observational studies. More human studies are needed to provide clear evidence of protective effects on health and to allow better evaluation of the potential risks of too-high polyphenol intake.

First, reliable food composition data are needed for estimation of the consumption of polyphenols, for study of their association with disease risk in an epidemiologic setting. This is a difficult task, because polyphenols in foods are not easy to measure, measurements are expensive, and the dataset will be large if all different compounds are included. Second, valid biomarkers should be identified, if possible, to provide a more independent estimate of the intake and metabolism of polyphenols. Some work on food composition tables and biomarkers is currently underway, as noted during this conference, but not enough well-designed, epidemiologic, observational studies have yet been performed. Much work on the bioavailability of polyphenols is being performed, which is an important step in the possible “rise of polyphenols.” The results of these studies should lead to additional investigations, because epidemiologic studies should be focused on polyphenols that are effectively bioavailable. If observational studies indicate possible beneficial effects of all or specific polyphenols, then small intervention trials should be started with either polyphenol supplements or specific foods.
especially rich in the compound of interest, with the use of surrogate endpoints such as established cardiovascular and cancer risk factors, intima/media thickness, and flow-mediated dilation. It will be important to take genetic factors into account in such studies. It is possible that some people are more or less sensitive to potential beneficial or deleterious effects of polyphenols. If all of these types of studies support a protective effect, then large-scale, population-based, intervention studies should be launched to determine whether there is a true causal beneficial effect. However, these will occur in the distant future, because such studies represent the ultimate verification of effects.

In conclusion, we should take into consideration the lessons of the story of β-carotene. We need to collect enough information from experimental, clinical, and epidemiologic research before we can support any specific recommendations regarding polyphenols (in particular, which polyphenols, at which doses, to achieve which benefits for which populations). To do this, we need more research, more funds, more patience, and more exchanges within the scientific community and among all research disciplines (basic and applied research). If we are not rigorous in finding clear answers for all of the aforementioned points, then we run the risk of needing to renounce recommendations in the future, damaging the credibility of nutritional recommendations from a public health viewpoint.

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Polyphenols in foods are more complex than often thought1–3

Véronique Cheynier

ABSTRACT
Dietary polyphenols show a great diversity of structures, ranging from rather simple molecules (monomers and oligomers) to polymers. Higher-molecular-weight structures (with molecular weights of > 500) are usually designated as tannins, which refers to their ability to interact with proteins. Among them, condensed tannins (proanthocyanidins) are particularly important because of their wide distribution in plants and their contributions to major food qualities. All phenolic compounds are highly unstable and rapidly transformed into various reaction products when the plant cells are damaged (for instance, during food processing), thus adding to the complexity of dietary polyphenol composition. The polyphenol composition of plant-derived foods and beverages depends on that of the raw material used but also on the extraction process and subsequent biochemical and chemical reactions of plant polyphenols. The occurrence of specific tannin-like compounds (ie, thearubigins and theaflavins) arising from enzymatic oxidation is well documented in black tea. Various chemical reactions involving anthocyanins and/or flavanols have been demonstrated to occur during red wine aging. Current knowledge regarding the reaction mechanisms involved in some of these processes and the structures of the resulting products is reviewed. Their effects on organoleptic and nutritional quality are also discussed. Am J Clin Nutr 2005;81(suppl):223S–9S.

KEY WORDS Polyphenols, anthocyanins, tannins, reaction products, oxidation, food, wine, tea, organoleptic properties, color, astringency

INTRODUCTION
Phenolic compounds are responsible for major organoleptic characteristics of plant-derived foods and beverages, particularly color and taste properties. They are also reported to contribute to the health benefits associated with consumption of diets high in fruits and vegetables or plant-derived beverages (such as tea and wine).

Innumerable studies have been devoted to polyphenols, their occurrence in plants and plant-derived foods, and their effects on food quality. However, plant polyphenol composition is still poorly understood, because most studies have focused on specific classes of molecules that can be separated and assayed with HPLC and neglected polymers that are not as easily determined. Furthermore, polyphenols are highly reactive compounds and good substrates for various enzymes, including polyphenoloxidases, peroxidases, glycosidases, and esterases. They undergo numerous enzymatic and chemical reactions during postharvest food storage and processing. Although the occurrence of such reactions and their roles in the development or degradation of food quality are well documented, the structures of the resulting products are still poorly understood and their concentrations in food are usually unknown. This article reviews current knowledge regarding the composition of plant-derived foods and beverages, with special emphasis on wine, which was taken as an example of a complex polyphenol–rich food product. The relationships between the structures and properties of genuine plant polyphenols and the reaction products derived from them are also briefly discussed.

POLYPHENOLS IN PLANTS
Plant polyphenols comprise a great diversity of compounds, among which flavonoids and several classes of nonflavonoids are usually distinguished (1). The latter (Figure 1) are mostly rather simple molecules, such as phenolic acids (which are subdivided into benzoic acids and hydroxycinnamic acids, based on C1–C6 and C3–C6 skeletons, respectively) and stilbenes, but also include complex molecules derived from them (eg, stilbene oligomers, gallotannins, ellagitannins, and lignins). The former (Figure 2) share a common nucleus consisting of 2 phenolic rings and an oxygenated heterocycle. They are divided into several groups differing in the oxidation state of the heterocyclic pyran ring (eg, anthocyanins, flavonols, and flavanols). More than 4000 flavonoids have been identified in plants, and the list is constantly growing (2). This is because of the occurrence of numerous substitution patterns in which primary substituents (eg, hydroxyl, methoxyl, or glycosyl groups) can themselves be substituted (eg, additionally glycosylated or acylated), sometimes yielding highly complex structures. Moreover, flavanols are also encountered as oligomers and polymers, referred to as condensed tannins or proanthocyanidins because they release anthocyanidins (ie, anthocyanin aglycones) when heated under acidic conditions. Proanthocyanidins differ in the nature of their constitutive units (eg, catechin and epicatechin in procyanidins, which release cyanidin after acid-catalyzed cleavage), their sequences, the positions of interflavanic linkages (C4-C6 or C4-C8 in the B-type series, with additional C2-O-C7 or C2-O-C5 bonds in A-type structures), their chain lengths, and the presence of substituents (eg, galloyl or glucosyl groups). Because all constitutive units and linkages can be distributed at random within a

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polymer, the number of possible isomers increases exponentially with the chain length [eg, 8 dimers, 32 trimers, and 128 tetramers in the B-type procyanidin series; more generally, \( n^x \cdot (n-1)^y \) for a polymer with \( x \) types of constitutive units and \( y \) types of linkages].

Plant polyphenol composition is highly variable both qualitatively and quantitatively; some of the compounds are ubiquitous, whereas others are restricted to specific families or species (eg, isoflavones in legumes). Polyphenol diversity in fruits and plant foods has been described in excellent reviews. Within a single species, large variations may also occur, particularly because of genetic factors, environmental conditions, and growth or maturation stages. For instance, in grapes and apples, anthocyanins are found only in the red varieties and accumulate toward the end of ripening. Pinot noir grapes contain only anthocyanin 3-glucosides, whereas most other grape cultivars also contain acylated anthocyanins. Major polyphenols in apples are hydroxycinnamic acids (mostly found as quinic esters, such as chlorogenic acid), flavonols (catechins and procyanidins), and dihydrochalcones; they are present in substantial amounts in flesh, but their concentrations in peel are higher. Flavonols and anthocyanins are present in smaller amounts and are almost completely restricted to peel. All of these compounds are constituents of most commonly consumed fruits except dihydrochalcones (eg, phloridzin), which are specific to apples.

Recent studies established that flavanols are the major polyphenols in apples, accounting for 65–85% of total polyphenol content. The remaining polyphenols are composed of flavonols, anthocyanins, and proanthocyanidins. Flavanols are known for their antioxidant and anti-inflammatory properties, which contribute to their health benefits.

**Figure 1.** Chemical structures of the main classes of nonflavonoid polyphenols.

**Figure 2.** Chemical structures of the main classes of flavonoids.
contents in the dessert (8) and cider (9) varieties analyzed. Their concentrations show large variations among apple cultivars, ranging from ~1 g/kg fresh weight in dessert varieties to 5 g/kg in cider varieties. Their chain lengths are also highly variable, averaging ~6 in all studied dessert varieties (8) but ranging up to 45 in some cider apple cultivars (9). Similarly, proanthocyanidins are the major polyphenols in grapes, where they are localized mostly in skins and seeds. Seed proanthocyanidins are partly galloylated procyanidins, with degrees of polymerization in the range of 1 (monomers) to ~20 (11). Skin proanthocyanidins contain both procyanidin and prodelphinidin units and are much larger than skin tannins (~30 units, on average) (12).

It is worth pointing out that difficulties in assaying flavanolic polymers, because of their heterogeneity (the large range of molecular weights and the increasing number of isomers as the chain length increases) and the lack of efficient separation techniques, have been overcome only recently. As a consequence, most compositional studies focused only on rather simple molecules, including monomers and flavanol oligomers, which can be individually quantified. In contrast, the concentrations of polymers have been determined in only a few species and the polymers are otherwise generally disregarded, although they are the major polyphenols in most plants (13).

Therefore, available food polyphenol composition data are limited, on the one hand, to the amounts of a limited number of molecules (eg, catechins) and, on the other hand, to approximate estimates of total polyphenol contents. The latter are based on absorbance measurements at 280 nm or the use of more or less specific reagents such as Folin Ciocalteu (assay based on an oxidation reaction), Bate-Smith reagent (proanthocyanidin assay based on the release of anthocyanidins after acid-catalyzed cleavage), or Porter’s reagent (vanillin-HCl, enabling detection of flavanols), which is often replaced with dimethylaminocinnamaldehyde. Although each of these methods can be used to compare samples with similar compositions, they are rather imprecise in assays of extracts with different polyphenol profiles, because of different responses of the various polyphenol structures and, for some, interference by nonphenolic compounds.

POLYPHENOLS IN PROCESSED FOODS

Overview

Polyphenols are highly unstable species that undergo numerous reactions in the course of food processing and storage. These changes are well known to food chemistry and technology specialists and have large effects on food quality. The resulting products account for large proportions of the polyphenolic contents in some foods and beverages, but they are overlooked in most studies addressing food composition. However, some of the new compounds formed in these processes may show particular properties different from those of their precursors.

An illustration of such changes can be found in a recent study comparing catechin contents of fresh and processed fruit and vegetables (14). Total catechin contents decreased by 28, 58, and 26% in rhubarb, broad beans, and cooking pears, respectively, after home preparation according to standard recipes. The concentrations measured in industrially canned products were also much lower than those in the equivalent fresh products.

Distribution in plant tissues and selective extraction

Changes associated with food processing involve selective extraction of some particular compounds with juice or wine technology but also removal of some parts (eg, peel and seeds) before consumption. For instance, hydroxycinnamic acids are the only phenolic compounds present in the flesh of most grape varieties (except teinturiers), whereas seeds contain galloylated procyanidins and skins contain flavonols and, in red varieties, anthocyanins, in addition to hydroxycinnamic acids and proanthocyanidins. Consequently, white grape juice made through direct pressing of the grapes contains only hydroxycinnamic acids and preparation of a red juice or wine requires a maceration step to extract red pigments from the skins. Monitoring of the phenolic composition of the fermenting red must enabled demonstration that anthocyanins diffuse faster than proanthocyanidins and that, among the latter, proanthocyanidins from skins that can be traced through their specific epigallocatechin units are extracted earlier than those from seeds, because of either their localization or their higher water solubility (15). Tannins with higher molecular weights also diffuse more slowly than oligomers. Extending the maceration time after the end of fermentation thus leads to increased extraction of proanthocyanidins and especially of higher-molecular-weight and highly galloylated structures. Higher-molecular-weight proanthocyanidins (beyond the decamer) have been reported to be insoluble in aqueous media. However, this has not been confirmed experimentally. In fact, polymers with average degrees of polymerization of > 20 were shown to be present in wine (16), and apple procyanidin fractions containing 70 units, on average, also proved to be soluble in wine-like hydroalcoholic medium (17).

Polyphenol reactions in food processing

Reactions involving polyphenols in food processing and storage include biochemical and chemical processes. The most important biochemical process is enzymatic oxidation, which starts as soon as the integrity of the cell is broken, but other types of enzymes, such as esterases, glycosidases, and decarboxylases, may also catalyze transformations and degradations of polyphenolic compounds. Enzymatic oxidation is ubiquitous in plant-derived foods. The resulting browning is usually detrimental to quality, particularly in postharvest storage of fresh fruits or juice and puree technology, but may be desirable for some products (eg, tea, coffee, cocoa, and raisins). Chemical reactions take place simultaneously and gradually become prevalent as the enzymatic activities decrease.

These mechanisms and the structures of the resulting products have been particularly studied in black tea and red wine. Enzymatic oxidation and subsequent reactions in black tea have been extensively reviewed (18, 19) and are not developed herein. Briefly, tea (Camelia sinensis L.) is particularly rich in polyphenols; they represent 30% of the leaf dry matter (18). Major polyphenols in the fresh tea leaves are flavanol monomers, among which (−)-epicatechin, (−)-epigallocatechin, and their gallic esters are particularly abundant. The so-called “fermentation” of black teas actually consists of enzymatic oxidation of native green tea polyphenols, catalyzed by polyphenoloxidase, followed by chemical reactions of the resulting quinones (18). A major part of tea leaf flavanols are thus converted into various types of products, including thearubigins and theaflavins, which are responsible for the characteristic dark brown color of black
Fractionation of the wine polyphenol extract with chromatography on a Fractogel column (TOSOH Biosep, Stuttgart, Germany) allowed separation of lower-molecular-weight polyphenols (fraction 1) from higher-molecular-weight polyphenols (fraction 2) (28, 29). Although fraction 1 was thought to consist mainly of simple polyphenolic species, its HPLC tracing at 520 nm showed a hump under the elution profile of grape anthocyanins, indicating that it contained other pigments (29). Extraction of this fraction with isoamyl alcohol, as proposed by Somers (27), allowed recovery of monomeric polyphenols in the organic phase, whereas unreolved oligomeric compounds were retained in the aqueous phase. The fraction 1 organic phase, fraction 1 aqueous phase, and fraction 2 each contained approximately one-third of the wine polyphenolic material.

Analysis of the fraction 1 aqueous phase and fraction 2 with HPLC after acid-catalyzed cleavage in the presence of toluene-α-thiol (thiolysis) showed that genuine proanthocyanidin units accounted for ~50% and 70%, respectively, of the material present. Average degrees of polymerization of 2.9 in the fraction 1 aqueous phase and 7.3 in fraction 2 were calculated, confirming that they were oligomeric and polymeric fractions, respectively. Color determinations indicated that anthocyanin derivatives represented ~6% of the polymeric material, whereas they accounted for 40% of polyphenols in the oligomeric fraction. Therefore, it seems that the major part of anthocyanin-tannin adducts are oligomeric rather than polymeric molecules. In addition, glucose determinations after hydrolysis, performed as described previously (30), indicated that a large proportion of anthocyanin derivatives were colorless, because most of the glucose released was assumed to be linked to flavonoid and particularly anthocyanin moieties. This suggests that anthocyanin-derived pigments exist both as red flavlyium forms and as colorless hydrated hemiketal forms, as demonstrated for genuine anthocyanins (31).

Analysis of the oligomeric fraction with HPLC coupled to ultraviolet/visible spectrophotometry and mass spectrometry showed the presence of 2 series of masses corresponding to anthocyanin-(epi)catechin adducts (29). The first group, in which the anthocyanin is in the red flavlyium form, presumably corresponds to flavanol-anthocyanin adducts (Figure 3, left). Such adducts were also obtained in wine-like model solutions containing anthocyanins and procyanidins. Their formation involves cleavage of the tannin interflavanic linkage, followed by nucleophilic addition of the anthocyanin hemiketal to the carbonation thus released and dehydration of the resulting adduct to the corresponding flavlyium (32). The second group (Figure 3, right) consists of colorless adducts in which the anthocyanin and flavanol units are linked by both carbon-carbon (C4-C8) and ether (C2-O-C7) bonds (A-type), formed by nucleophilic addition of the flavanol to the anthocyanin flavlyium cation (33). Both types of dimeric adducts were also released with thiolysis of the oligomeric and polymeric fractions, indicating that these moieties were also incorporated in higher-molecular-weight molecules.

Various other anthocyanin and tannin reactions have been shown to occur in wines. These include reactions involving acetaldehyde, arising from yeast metabolism or ethanol oxidation. Acetaldehyde-induced reactions yield ethyl-linked species, including flavanol anthocyanin adducts (34), flavanol oligomers (35), and anthocyanin oligomers (36), as well as another group of pigments based on a pyranoanthocyanin structure (37, 38) (Figure 4). Pyranoanthocyanins are formed through reactions of anthocyanins with compounds with a polarizable double bond, such as vinyl or enol derivatives. Addition of vinylphenol (39), pyruvic acid (40), or acetaldehyde (41, 42), which are yeast metabolites formed during fermentation, to anthocyanins takes place spontaneously in wine, yielding phenyl-pyranoanthocyanins (R = phenyl), carboxy-pyranoanthocyanins (R = COOH), and pyranoanthocyanins (R = H), respectively.
Other aldehydes, such as glyoxalic acid (resulting from oxidation of tartaric acid, which is present in wine in large amounts) or furfural (extracted from oak barrels), can replace acetaldehyde in polymerization reactions (43, 44). The adducts thus formed then proceed to new xanthylum salt pigments (45). Finally, degradation of anthocyanins releases lower-molecular-weight compounds after cleavage of the heterocyclic ring (eg, syringic acid from malvidin derivatives).

Despite the variety of mechanisms and the large number of products demonstrated, wine composition remains largely unknown. In fact, each of the elucidated derived structures is present in only small amounts and, taken together, they account for only a small proportion of wine polyphenols. It should be emphasized that all flavanols initially present can be similarly involved in all of these reactions, so that each of the flavanol derivatives detected is in fact the head of a large family. In addition, although most new pigments identified are based on malvidin 3-glucoside, the major anthocyanin in grapes, other anthocyanins are expected to react in the same way, yielding a series of derivatives. Among the anthocyanin derivatives formed in wine, pyrananthocyanins are orange pigments, whereas ethyl-linked species and tannin-anthocyanin adducts are purple. All 3 types of molecules are more resistant to discoloration through hydration reactions than are genuine grape anthocyanins. It was recently shown that ethyl-linked anthocyanin dimers in wine are in a single form, in which one anthocyanin unit is colorless and the other is red (51). This means that conversion of grape pigments (75–80% colorless in the wine pH range) to ethyl-linked dimers (one colorless unit and one red unit, ie, 50% colorless) not only leads to a slight shift from red to purple but also greatly enhances color intensity and stability. Reactions of anthocyanins also yield colorless products, such as A-type anthocyanin-flavanol adducts (33) and syringic acid, whereas ethyl-linked species and tannin-anthocyanin adducts are purple. All 3 types of molecules are more resistant to discoloration through hydration reactions than are genuine grape anthocyanins.

Other major polyphenol pigments in plants are anthocyanins, which exhibit red, purple, or blue color, and, to a lesser extent, the yellow flavonols and flavones. Anthocyanins are highly reactive species. When dissolved in water, the anthocyanin red flavylium cations are converted to several other forms through proton transfer and hydration reactions (31, 50). In slightly acidic media such as encountered in plant foods, simple anthocyanins are present mostly in the colorless hemiketal form, in equilibrium with the yellow chalcone isomer (31). Conversion of genuine anthocyanins to other molecules during the course of food processing results in either loss or stabilization of color and increases the range of available hues. Among the anthocyanin derivatives formed in wine, pyrananthocyanins are orange pigments, whereas ethyl-linked species and tannin-anthocyanin adducts are purple.

PROPERTIES OF FOOD POLYPHENOLOLS

Polyphenols exhibit a wide range of properties, depending on their particular structures. They include yellow, orange, red, and blue pigments, as well as various compounds involved in food flavor. Some volatile polyphenols, such as vanillin and eugenol (which is responsible for the characteristic odor of cloves), are extremely potent odorants, but the major flavors associated with polyphenols are bitterness and astringency. Other major polyphenol characteristics include their radical-scavenging capacity, which is involved in antioxidant properties, and their ability to interact with proteins. The latter is responsible for astringency perception (resulting from interactions of tannins with salivary proteins), for formation of haze and precipitates in beverages, and for inhibition of enzymes and reduced digestibility of dietary proteins.

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Other major polyphenol properties, such as the ability to complex with proteins and free radical-scavenging capacity, are primarily related to the number and accessibility of phenol (in particular, o-diphenol) moieties. The oxygen radical-scavenging capacity of procyanidin dimers and trimers was shown to increase with galloylation and to a lesser extent with longer chain
length but was also influenced by the position of galloyl substituents (52). Similar results were obtained for scavenging of the radical cation 2,2′-azinobis(3-ethyl-benzothiazoline-6-sulfonate) in the aqueous phase, but antioxidant activity decreased from the trimer to the tetramer. Conversely, in the lipid phase, radical-scavenging activity decreased as the molecular weight increased (53). These conflicting results suggest that antioxidant effects are exerted through different mechanisms in the different assays. One should be careful in assessing the relevance of in vitro tests as predictors of in vivo situations.

The affinity of proanthocyanidins for proteins (54) and their astringency (17) increase with both the degree of polymerization and the extent of galloylation. Complex transformation products of plant polyphenols can be similarly expected to act as radical scavengers and bind to proteins. Very few studies have addressed this issue, because of the difficulty of isolating the compounds in sufficient amounts. Flavanol dimers arising from catechin oxidation showed enzyme inhibition effects similar to those of their procyanidin isomers, whereas yellow products obtained after another oxidation step were more active (55). It is not known whether other tannin-like molecules, such as ethyl- or ethanoic-linked flavanol oligomers, behave like tannins, but conversion of grape tannins to new products, particularly tannin-anthocyanin adducts, is generally reported to reduce wine astringency (27). This was classically ascribed to an increase in molecular weight, because larger tannins were thought to be insoluble and thus non-astringent. However, recent studies showed that higher-molecular-weight proanthocyanidins are both soluble and more astringent than the oligomeric proanthocyanidins (17). Consequently, the decrease in astringency observed during wine aging is likely to involve acid-catalyzed processes leading to lower-molecular-weight species, as described above, rather than polymerization reactions. However, the taste of polyphenol reactions products and the effect on astringency of incorporating anthocyanin units into a tannin structure remain to be investigated.

Properties of polyphenols are also greatly affected by their interactions with other constituents of the food matrix. Color intensification resulting from interactions of anthocyanins with other compounds (ie, copigmentation) is well documented (56, 57). The astringency of tannins may also be altered by the presence of various molecules, including polysaccharides and proteins. A mechanism involving interactions of tannins with soluble pectins released during ripening, impeding their binding to salivary proteins, has been proposed to explain changes that occur during fruit ripening (58). The formation of soluble and colloidal polysaccharide-tannin complexes in wine-like model systems was demonstrated with light-scattering measurements (59). Similarly, analyses of wines before and after protein fining suggested that the reduction of astringency induced by fining was partly attributable to the incorporation of tannins in soluble tannin-protein complexes.

Many products arising from various reaction pathways have been determined in different foods and beverages, but their composition is still largely unknown. The properties of complex genuine polyphenols and derived molecules also remain to be established. Additional important questions are related to their bioavailability. In fact, absorption of complex structures in the small intestine, as described for phenolic acids, flavan monomers, and flavonols, and their circulation in conjugated forms appears very unlikely. However, they may be metabolized by the gut microflora, yielding the same lower-molecular-weight metabolites as those arising from genuine polyphenols and also possibly others. The new linkages formed include labile ones that should not resist the digestive process but also extremely strong ones. For example, catechin dimers arising from enzymatic oxidation contain extremely resistant interflavanic linkages (60). Their fermentation by the digestive flora should consequently yield both metabolites identical to those obtained from catechin or procyanidins after cleavage of the heterocyclic ring and additional metabolites derived from the biphenyl moiety. Finally, strong interactions with other constituents of the food matrix are likely to interfere with the metabolism of polyphenols and should be taken into account in bioavailability studies. Indeed, interactions of polyphenols with food proteins and digestive enzymes are well known to reduce protein digestibility and can be expected to alter polyphenol bioavailability similarly.

REFERENCES
Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies\textsuperscript{1–3}

Claudine Manach, Gary Williamson, Christine Morand, Augustin Scalbert, and Christian Rémésy

ABSTRACT

Polyphenols are abundant micronutrients in our diet, and evidence for their role in the prevention of degenerative diseases is emerging. Bioavailability differs greatly from one polyphenol to another, so that the most abundant polyphenols in our diet are not necessarily those leading to the highest concentrations of active metabolites in target tissues. Mean values for the maximal plasma concentration, the time to reach the maximal plasma concentration, the area under the plasma concentration-time curve, the elimination half-life, and the relative urinary excretion were calculated for 18 major polyphenols. We used data from 97 studies that investigated the kinetics and extent of polyphenol absorption among adults, after ingestion of a single dose of polyphenol provided as pure compound, plant extract, or whole food/beverage. The metabolites present in blood, resulting from digestive and hepatic activity, usually differ from the native compounds. The nature of the known metabolites is described when data are available. The plasma concentrations of total metabolites ranged from 0 to 4 \( \mu \text{mol/L} \) with an intake of 50 mg aglycone equivalents, and the relative urinary excretion ranged from 0.3\% to 43\% of the ingested dose, depending on the polyphenol. Gallic acid and isoflavones are the most well-absorbed polyphenols, followed by catechins, flavanones, and quercetin glucosides, but with different kinetics. The least well-absorbed polyphenols are the proanthocyanidins, the galloylated tea catechins, and the anthocyanins. Data are still too limited for assessment of hydroxycinnamic acids and other polyphenols. These data may be useful for the design and interpretation of intervention studies investigating the health effects of polyphenols. Am J Clin Nutr 2005;81(suppl):230S–42S.

KEY WORDS Polyphenols, flavonoids, isoflavones, flavanols, flavanones, hydroxycinnamic acids, hydroxybenzoic acids, anthocyanins, proanthocyanidins, catechins, bioavailability, metabolism, pharmacokinetics, elimination half-life, humans

INTRODUCTION

Epidemiologic studies have clearly shown that diets rich in plant foods protect humans against degenerative diseases such as cancer and cardiovascular diseases. Plant foods contain fiber, vitamins, phytosterols, sulfur compounds, carotenoids, and organic acids, which contribute to the health effects, but they also contain a variety of polyphenols, which are increasingly regarded as effective protective agents.

Polyphenols represent a wide variety of compounds, which are divided into several classes, ie, hydroxybenzoic acids, hydroxycinnamic acids, anthocyanins, proanthocyanidins, flavonols, flavones, flavanols, flavanones, isoflavones, stilbenes, and lignans.

The chemical structures and the food contents of the various polyphenols have been reviewed elsewhere (1). One of the main objectives of bioavailability studies is to determine, among the hundreds of dietary polyphenols, which are better absorbed and which lead to the formation of active metabolites.

Many researchers have investigated the kinetics and extent of polyphenol absorption by measuring plasma concentrations and/or urinary excretion among adults after the ingestion of a single dose of polyphenol, provided as pure compound, plant extract, or whole food/beverage. We have reviewed 97 studies of various classes of polyphenols, namely, anthocyanins, flavonols, flavanones, flavanol monomers, proanthocyanidins, isoflavones, hydroxycinnamic acids, and hydroxybenzoic acids. We have compiled the data from the most relevant studies, ie, those using well-described polyphenol sources and accurate methods of analysis, to calculate mean values for several bioavailability measures, including the maximal plasma concentration (\( C_{\text{max}} \)), time to reach \( C_{\text{max}} \) area under the plasma concentration-time curve, elimination half-life, and relative urinary excretion. The results clearly show wide variability in the bioavailability of the different polyphenols.

ANTHOCYANINS

Anthocyanins are present in very large amounts in some diets. Servings of 200 g of aubergine or black grapes can provide up to 1500 mg anthocyanins and servings of 100 g of berries up to 500 mg. Therefore, an intake of several hundred milligrams would not be considered exceptional. The mean dietary intake in Finland has been estimated to be 82 mg/d, with the main sources being berries, red wine, juices, and the coloring agent E163 (M Heinonen, personal communication, 2001).

The results of a literature survey on the bioavailability of anthocyanins among humans are presented in Table 1. Single doses of 150 mg to 2 g total anthocyanins were given to the volunteers, generally in the form of berries, berry extracts, or concentrates. After such intakes, concentrations of anthocyanins...
measured in plasma were very low, on the order of 10–50 nmol/L. The mean time to reach Cmax was 1.5 h (range: 0.75–4 h) for most studies reported low relative urinary excretions, ranging from 0.004% to 0.1% of the intake, although Lapidot et al (11) and Felgines et al (14) measured higher levels of anthocyanin excretion (up to 5%) after red wine or strawberry consumption. The time course of absorption was consistent with absorption in the stomach, as described for animals (15, 16). The most striking features of the survey were thus that anthocyanins are very rapidly absorbed and eliminated and that they are absorbed with poor efficiency.

Although anthocyanin bioavailability appears low, it could have been underestimated, for 2 main reasons, ie, some important metabolites might have been ignored or the methods used might need to be optimized for the analysis of anthocyanin metabolites. It is well known that different chemical forms of anthocyanins are present in equilibrium, depending on the pH. In most studies, analyses were performed with ultraviolet-visible light detection, on the basis of complete conversion of all of the chemical forms of anthocyanins into a colored flavylum cation with acidification. However, it is possible that some forms existing at neutral pH would not be converted into the flavylum form, because of putative binding to or chemical reactions with other components of the plasma or urine, for example. It would be very useful to have labeled anthocyanins for identification of all of the metabolites generated from these polyphenols.

With our current knowledge, there seem to be important differences in the metabolism of anthocyanins, compared with other polyphenols. Whereas flavonoids are generally recovered in plasma and urine as glucuronidated and/or sulfated derivatives, with no or only trace amounts of native forms, unchanged glycosides were the only metabolites identified for anthocyanins in most studies. However, glucuronides and sulfates of anthocyanins were recently identified in human urine with HPLC-mass spectrometry/mass spectrometry analyses (6, 14). In the study conducted by Felgines et al (14), monoglucuronides accounted for >80% of the total metabolites when analyses were performed immediately after urine collection. The authors also showed that all of the metabolites of the strawberry anthocyanins, except for the native glucoside, were very unstable and were extensively degraded when acidified urine samples were frozen for storage. This probably explains why such metabolites were not observed in previous studies. Therefore, it seems crucial to reconsider anthocyanin bioavailability, with methods that allow preservation of all of the metabolites in frozen samples.

Other metabolites that have not yet been considered but could contribute to the biological effects of anthocyanins are the metabolites produced by the intestinal microflora. However, studies performed in the 1970s showed that degradation of anthocyanins by the microflora occurs to a much more limited extent than with other flavonoids (17). Protocatechuic acid was identified as an abundant metabolite of cyanidin-3-O-glucoside in rats, but it was also formed in vitro with simple incubation of cyanidin with rat plasma in the absence of colonic bacteria (18). Identification of all of the microbial metabolites in humans should be reinvestigated with pure anthocyanins and not only berry extracts, which contain other polyphenols as well as anthocyanins.

### FLAVONOLS

Flavonols, especially quercetin, have been extensively studied, mainly because they are widely distributed in dietary plants. However, their content in the diet is generally quite low. The daily intake of flavonols has been estimated as only 20–35 mg/d (19–22).

Twenty years after Gugler et al (23, 24) failed to find quercetin in plasma or urine from volunteers challenged with 4 g pure aglycone, the team of Hollman et al (23, 24) showed that quercetin was indeed absorbed in humans. They demonstrated that glucosides of quercetin were more efficiently absorbed than quercetin itself, whereas the rhamnoglucoside (rutin) was less efficiently and less rapidly absorbed (Table 2). This difference in absorption rates was confirmed by others (33, 34). When pure compounds were given, the bioavailability of rutin was ~20%
that of quercetin glucosides, on the basis of area under the plasma concentration-time curve values and relative urinary excretions (30, 34). The biochemical explanation for the better absorption of quercetin glucosides has been discussed elsewhere (1). It is clear that, for quercetin, bioavailability differs among food sources, depending on the type of glycosides they contain. For example, onions, which contain glucosides, are better sources of bioavailable quercetin than are apples and tea, which contain rutin and other glycosides.

The presence of intact glycosides of quercetin in plasma was debated a few years ago, but it is now accepted that such compounds are absent from plasma after nutritional doses (34, 37–40). Quercetin is not present as an aglycone and occurs only in conjugated forms. Generally, ∼20–40% of quercetin is methylated in the 3'-position, yielding isorhamnetin (31, 34, 38). The exact nature of the metabolites present in plasma after the ingestion of onions was determined by Day et al (38). They identified quercetin-3-O-glucuronide, 3'-O-methylquercetin-3-O-glucuronide, and quercetin-3'-O-sulfate as the major conjugates.

Some phenolic and aromatic acids can also be produced from flavonols by the microflora. Quercetin degradation produces mainly flavanols and flavones. Quercetin degradation produces mainly flavanones by the microflora, before absorption. Flavanones represent a small group of compounds, including glycosides of hesperetin present in oranges and glycosides of naringenin present in grapefruit. The bioavailability of the glycosides of eriodictyol, present in lemons, has never been studied (37). It should be noted that very high interindividual variability was observed in the latter study and in others (27, 34, 37). Some individuals could be better absorbers than others, possibly because of particular polymorphisms for intestinal enzymes or transporters. Quantitative data are still lacking for other flavonols and flavones.

### TABLE 2

Bioavailability studies of flavonol- or flavanol-containing foods

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of subjects</th>
<th>Dose</th>
<th>T(_{\text{max}}) plasma</th>
<th>Plasma concentration</th>
<th>AUC</th>
<th>Urinary excretion</th>
<th>Elimination half-life</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure quercetin</td>
<td>6</td>
<td>4 g</td>
<td></td>
<td>h μmol/L</td>
<td>0.14–0.42</td>
<td>5.0–18.8</td>
<td>21.6–72</td>
<td></td>
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<tr>
<td>Onions</td>
<td>9 ileostomized</td>
<td>89 mg quercetin eq</td>
<td></td>
<td>&lt;0.33 μmol/L</td>
<td>7.3–18.8</td>
<td>32.1–83.1</td>
<td>10.9–34</td>
<td></td>
</tr>
<tr>
<td>Pure rutin</td>
<td>9 ileostomized</td>
<td>100 mg quercetin eq</td>
<td></td>
<td>&lt;0.33 μmol/L</td>
<td>7.3–18.8</td>
<td>32.1–83.1</td>
<td>10.9–34</td>
<td></td>
</tr>
<tr>
<td>Pure quercetin</td>
<td>9 ileostomized</td>
<td>100 mg quercetin eq</td>
<td></td>
<td>&lt;0.33 μmol/L</td>
<td>7.3–18.8</td>
<td>32.1–83.1</td>
<td>10.9–34</td>
<td></td>
</tr>
<tr>
<td>Fried onions</td>
<td>2</td>
<td>64 mg quercetin eq</td>
<td></td>
<td>0.65 μmol/L</td>
<td>1.3–1.9</td>
<td>4.5–17.5</td>
<td>17.7–33</td>
<td></td>
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<tr>
<td>Onions</td>
<td>9</td>
<td>68 mg quercetin eq</td>
<td></td>
<td>0.74 μmol/L</td>
<td>5.0–7.3</td>
<td>1.8–3.6</td>
<td>28.1–30</td>
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<tr>
<td>Apples</td>
<td>9</td>
<td>107 mg quercetin eq</td>
<td></td>
<td>0.3 μmol/L</td>
<td>2.2–3.7</td>
<td>3.5–10.3</td>
<td>23.0–26</td>
<td></td>
</tr>
<tr>
<td>Pure rutin</td>
<td>9</td>
<td>100 mg quercetin eq</td>
<td></td>
<td>0.3 μmol/L</td>
<td>3.5–10.3</td>
<td>23.0–26</td>
<td>23.0–26</td>
<td></td>
</tr>
<tr>
<td>Complete meal</td>
<td>10</td>
<td>87 mg quercetin eq</td>
<td></td>
<td>0.37 μmol/L</td>
<td>2.1–4.0</td>
<td>3.3–10.3</td>
<td>23.0–26</td>
<td></td>
</tr>
<tr>
<td>Onions</td>
<td>5</td>
<td>186 mg quercetin eq</td>
<td></td>
<td>1.3–1.9 μmol/L</td>
<td>2.1–4.0</td>
<td>3.3–10.3</td>
<td>23.0–26</td>
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<tr>
<td>Onions</td>
<td>5</td>
<td>50 mg quercetin eq</td>
<td></td>
<td>0.83 μmol/L</td>
<td>2.1–4.0</td>
<td>3.3–10.3</td>
<td>23.0–26</td>
<td></td>
</tr>
<tr>
<td>Quercetin 4'-glucoside</td>
<td>9</td>
<td>150 mg</td>
<td></td>
<td>0.5 μmol/L</td>
<td>0.14–0.5</td>
<td>1.7–4.5</td>
<td>21.6–30</td>
<td></td>
</tr>
<tr>
<td>Pure rutin</td>
<td>9</td>
<td>190 mg</td>
<td></td>
<td>0.18 μmol/L</td>
<td>0.1–0.5</td>
<td>1.7–4.5</td>
<td>21.6–30</td>
<td></td>
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<tr>
<td>Quercetin 3-glucoside</td>
<td>9</td>
<td>156 mg</td>
<td></td>
<td>5 μmol/L</td>
<td>4.5–12.6</td>
<td>3.5–10.3</td>
<td>23.0–26</td>
<td></td>
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<tr>
<td>Quercetin 4'-glucoside</td>
<td>9</td>
<td>160 mg</td>
<td></td>
<td>4.5 μmol/L</td>
<td>17.5–27.5</td>
<td>3.1–10.3</td>
<td>23.0–26</td>
<td></td>
</tr>
<tr>
<td>Pure rutin</td>
<td>3</td>
<td>500 mg</td>
<td></td>
<td>4–7 μmol/L</td>
<td>0.13–0.73</td>
<td>2.9–7.5</td>
<td>3.0–11.8</td>
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<tr>
<td>Pure quercetin</td>
<td>16</td>
<td>8, 20, 50 mg quercetin eq</td>
<td></td>
<td>0.14, 0.22, 0.29</td>
<td>1.74, 2.92, 3.77</td>
<td>17, 17.7, 15 %</td>
<td>33, 34, 37</td>
<td></td>
</tr>
<tr>
<td>Pure rutin</td>
<td>16</td>
<td>8, 20, 50 mg quercetin eq</td>
<td></td>
<td>0.14, 0.22, 0.29</td>
<td>1.74, 2.92, 3.77</td>
<td>17, 17.7, 15 %</td>
<td>33, 34, 37</td>
<td></td>
</tr>
<tr>
<td>Onions</td>
<td>12</td>
<td>100 mg quercetin eq</td>
<td></td>
<td>0.06–0.18</td>
<td>1.26, 2.10, 3.36</td>
<td>13, 23, 36</td>
<td>33, 34, 37</td>
<td></td>
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<tr>
<td>Pure rutin</td>
<td>12</td>
<td>100 mg quercetin eq</td>
<td></td>
<td>0.06–0.18</td>
<td>1.26, 2.10, 3.36</td>
<td>13, 23, 36</td>
<td>33, 34, 37</td>
<td></td>
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<tr>
<td>Buckwheat tea</td>
<td>12</td>
<td>200 mg quercetin eq</td>
<td></td>
<td>0.18–0.3</td>
<td>1.26, 2.10, 3.36</td>
<td>13, 23, 36</td>
<td>33, 34, 37</td>
<td></td>
</tr>
<tr>
<td>Pure rutin</td>
<td>12</td>
<td>200 mg quercetin eq</td>
<td></td>
<td>0.18–0.3</td>
<td>1.26, 2.10, 3.36</td>
<td>13, 23, 36</td>
<td>33, 34, 37</td>
<td></td>
</tr>
<tr>
<td>Apple cider (1.1 L)</td>
<td>6</td>
<td>1.6 mg quercetin eq</td>
<td></td>
<td>0.66–1 μmol/L</td>
<td>3.5–10.3</td>
<td>17.7–33</td>
<td>33, 34, 37</td>
<td></td>
</tr>
<tr>
<td>Pure quercetin</td>
<td>12</td>
<td>0.14 mg/kg/bw</td>
<td></td>
<td>0.15–0.42</td>
<td>2.9–7.5</td>
<td>3.0–11.8</td>
<td>33, 34, 37</td>
<td></td>
</tr>
</tbody>
</table>

\(^{1}\)T\(_{\text{max}}\): time to C\(_{\text{max}}\); AUC, area under the curve; eq, equivalents; bw, body weight.
contains naringenin aglycone. However, natural foods rarely contain significant amounts of flavanones in the aglycone form. Plasma metabolites of flavanones have not yet been identified. Monoglucuronides of hesperetin were shown to be the major forms present in plasma after ingestion of orange juice, but the positions of glucuronidation are still not known (48). Microbial metabolites such as p-hydroxyphenylpropionic acid, p-coumaric acid, p-hydroxybenzoic acid, and phenylpropionic acid were produced with in vitro incubation of naringenin with human microflora (17, 55, 56). They were also detected in rat urine (57). The same types of microbial metabolites were detected for hesperetin (58, 59). Therefore, microbial metabolites may also be present in human plasma.

The total urinary excretion of conjugated flavanones accounted for 8.6% of the intake for hesperidin and 8.8% for naringin (Table 3). Plasma concentrations reached 1.3–2.2 μmol/L hesperetin metabolites with an intake of 130–220 mg given as orange juice (48, 49) and up to 6 μmol/L naringenin metabolites with 200 mg ingested as grapefruit juice (49). However, data are still scarce, with only 3 studies having investigated the bioavailability of flavanones in plasma.

### TABLE 3
Bioavailability studies of flavanones or flavanone-containing foods

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of subjects</th>
<th>Dose</th>
<th>Tmax (h)</th>
<th>Plasma concentration (μmol/L)</th>
<th>AUC (μmol·h/L)</th>
<th>Urinary excretion (% of intake)</th>
<th>Elimination half-life (h)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange juice</td>
<td>5</td>
<td>110 or 220 mg eq hesperetin</td>
<td>5.4–5.8</td>
<td>0.46–1.28</td>
<td>4.19–9.28</td>
<td>4.1–6.4</td>
<td>7.1–7.8</td>
<td>48</td>
</tr>
<tr>
<td>Orange juice</td>
<td>5</td>
<td>22.6 or 45 mg eq naringenin</td>
<td>4.6–5</td>
<td>0.06–0.2</td>
<td>0.43–1.29</td>
<td>0.71–1.8</td>
<td>2.7–5</td>
<td>48</td>
</tr>
<tr>
<td>Orange juice</td>
<td>8</td>
<td>126 mg eq hesperetin</td>
<td>5.4</td>
<td>2.2</td>
<td>10.3</td>
<td>5.3</td>
<td>2.2</td>
<td>49</td>
</tr>
<tr>
<td>Orange juice</td>
<td>8</td>
<td>23 mg eq naringenin</td>
<td>5.5</td>
<td>0.64</td>
<td>2.6</td>
<td>1.1</td>
<td>1.3</td>
<td>49</td>
</tr>
<tr>
<td>Grapefruit juice</td>
<td>5</td>
<td>199 mg eq naringenin</td>
<td>4.8</td>
<td>5.99</td>
<td>27.7</td>
<td>30.2</td>
<td>2.2</td>
<td>49</td>
</tr>
<tr>
<td>Tomato paste</td>
<td>5</td>
<td>3.8 mg naringenin</td>
<td>2</td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Pure compound</td>
<td>6</td>
<td>500 mg pure naringin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>Pure compound</td>
<td>1</td>
<td>500 mg pure naringin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>52</td>
</tr>
<tr>
<td>Pure compound</td>
<td>1</td>
<td>500 mg pure hesperidin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>52</td>
</tr>
<tr>
<td>Grapefruit juice</td>
<td>4</td>
<td>325 mg eq naringenin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>52</td>
</tr>
<tr>
<td>Orange juice</td>
<td>4</td>
<td>44 mg eq hesperetin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>52</td>
</tr>
<tr>
<td>Grapefruit juice</td>
<td>6</td>
<td>7.2 mg naringin/kg bw</td>
<td></td>
<td>8.9</td>
<td>2.6–2.9</td>
<td>53</td>
<td></td>
<td>53</td>
</tr>
<tr>
<td>Grapefruit juice</td>
<td>2</td>
<td>214 mg naringin/d for 1 wk</td>
<td></td>
<td>14d</td>
<td></td>
<td></td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

1 Tmax, time to Cmax; AUC, area under the curve; eq, equivalents; bw, body weight.
### TABLE 4

Bioavailability studies of flavanols or flavanol-containing foods

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of subjects</th>
<th>Dose</th>
<th>T&lt;sub&gt;max&lt;/sub&gt;</th>
<th>Plasma concentration</th>
<th>AUC</th>
<th>Urinary excretion</th>
<th>Elimination half-life</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocoa beverage</td>
<td>5</td>
<td>323 mg catechins</td>
<td>2</td>
<td>5.9 EC + 0.16 catechins</td>
<td></td>
<td></td>
<td></td>
<td>62</td>
</tr>
<tr>
<td>Chocolate (80 g)</td>
<td>10</td>
<td>137 mg EC</td>
<td>2</td>
<td>0.26</td>
<td></td>
<td></td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>Cocoa</td>
<td>6</td>
<td>1.53 mg/kg bw</td>
<td>2</td>
<td>1–1.5</td>
<td></td>
<td></td>
<td>1.7–3</td>
<td>64</td>
</tr>
<tr>
<td>Cocoa</td>
<td>5</td>
<td>220 mg EC</td>
<td>2</td>
<td>4.92</td>
<td></td>
<td></td>
<td>25.3</td>
<td>65</td>
</tr>
<tr>
<td>Chocolate</td>
<td>5</td>
<td>220 mg EC</td>
<td>2</td>
<td>4.77</td>
<td></td>
<td></td>
<td>29.8</td>
<td>65</td>
</tr>
<tr>
<td>Chocolate</td>
<td>20</td>
<td>46, 92, 138 mg EC</td>
<td>2</td>
<td>0.13, 0.26, 0.36</td>
<td></td>
<td></td>
<td></td>
<td>66</td>
</tr>
<tr>
<td>Chocolate (40, 80 g)</td>
<td>8</td>
<td>82, 164 mg EC</td>
<td>2–2.6</td>
<td>0.38, 0.7</td>
<td>1.53, 3.7</td>
<td></td>
<td>1.9–2.3</td>
<td>67</td>
</tr>
<tr>
<td>Red wine (120 mL)</td>
<td>9</td>
<td>35 mg catechin</td>
<td>1.5</td>
<td>0.091</td>
<td>0.36</td>
<td></td>
<td>3.1</td>
<td>68</td>
</tr>
<tr>
<td>Red wine (120 mL)</td>
<td>9</td>
<td>35 mg catechin</td>
<td>1.44</td>
<td>0.077</td>
<td>0.31</td>
<td></td>
<td>3.2</td>
<td>69</td>
</tr>
<tr>
<td>Red wine (120 mL)</td>
<td>9</td>
<td>35 mg catechin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3–10</td>
<td>70</td>
</tr>
<tr>
<td>Pure catechin</td>
<td>12</td>
<td>0.36 mg/kg bw</td>
<td>0.5</td>
<td>0.14–0.49</td>
<td>2.36–28.2</td>
<td></td>
<td>1–1.3</td>
<td>72</td>
</tr>
<tr>
<td>Pure catechin</td>
<td>3</td>
<td>2 g</td>
<td>2–3</td>
<td>2.8–5.9</td>
<td>22–37</td>
<td></td>
<td>55.0</td>
<td>71</td>
</tr>
<tr>
<td>Pure catechin</td>
<td>6</td>
<td>0.5, 1, 2 g</td>
<td>1.4–2</td>
<td>2.3, 8.7</td>
<td>4.5, 9.7, 20.1</td>
<td></td>
<td>23.6–28.2</td>
<td>72</td>
</tr>
<tr>
<td>Pure EGCG</td>
<td>6 × 8</td>
<td>50, 100, 200, 400, 800, 1600 mg</td>
<td>1.3–2.2</td>
<td>0.28, 0.39, 0.72, 1.36, 2.33, 7.4 EGCG</td>
<td>0.9, 2.6, 2.7, 5.5, 8.3, 22.4</td>
<td></td>
<td>1.9–4.6</td>
<td>73</td>
</tr>
<tr>
<td>Pure EGCG</td>
<td>4</td>
<td>2 mg/kg bw</td>
<td>2</td>
<td>0.097 EGCG + 0.018</td>
<td>0.52 EGCG + 4', 4'diMe EGCG</td>
<td>0.1 diMe EGCG</td>
<td>2.5 EGCG, 2.8', 4'diMe EGCG</td>
<td>74</td>
</tr>
<tr>
<td>Pure EGCG</td>
<td>8</td>
<td>2 mg/kg bw</td>
<td>1.6</td>
<td>0.075 EGCG</td>
<td></td>
<td></td>
<td>0.47</td>
<td>75</td>
</tr>
<tr>
<td>Pure EGCG</td>
<td>4 × 5</td>
<td>200, 400, 600, 800 mg</td>
<td>1.8–4</td>
<td>0.16, 0.24, 0.37, 0.96 EGCG</td>
<td>0.8, 1.3, 3.7, 6.1</td>
<td></td>
<td>1.9–3.1</td>
<td>76</td>
</tr>
<tr>
<td>Polyphenon E</td>
<td>4 × 5</td>
<td>200, 400, 600, 800 mg</td>
<td>2.4–4.1</td>
<td>0.16, 0.27, 0.36, 0.82 EGCG</td>
<td>0.8, 1.9, 2.9, 5.9</td>
<td></td>
<td>1.9–3.6</td>
<td>76</td>
</tr>
<tr>
<td>Green tea powder</td>
<td>4</td>
<td>105 mg EGCG</td>
<td>2</td>
<td>0.14–0.31 EGCG</td>
<td></td>
<td></td>
<td></td>
<td>77</td>
</tr>
<tr>
<td>Pure EGCG</td>
<td>10</td>
<td>688 mg</td>
<td>2.9</td>
<td>1.3 EGCG</td>
<td>12.1</td>
<td>&lt;0.02</td>
<td></td>
<td>78</td>
</tr>
<tr>
<td>Pure EGCG</td>
<td>4</td>
<td>459 mg</td>
<td>1.7</td>
<td>5 EGCG + 1.9 Me EGC</td>
<td>20.1 EGCG + 12.6 Me EGC</td>
<td>39.9</td>
<td>9.8 EC + 3.8 Me EGC</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Pure EC gallate</td>
<td>10</td>
<td>663 mg</td>
<td>4</td>
<td>3.1 EG gallate</td>
<td>0.66, 4.3, 4.4 EGCG at 1.5 h</td>
<td>0.03, 0.14, 0.25 EGC at 1.5 h</td>
<td>1.7 EGCG, 2.5 Me EGC</td>
<td>6.9</td>
</tr>
<tr>
<td>Green tea extract</td>
<td>3</td>
<td>225, 375, 525 mg EGCG</td>
<td>7.5, 12.5, 17.5 mg EGCG</td>
<td>0.66, 4.3, 4.4 EGCG at 1.5 h</td>
<td>0.03, 0.14, 0.25 EGC at 1.5 h</td>
<td>1.7 EGCG, 2.5 Me EGC</td>
<td>6.9</td>
<td>79</td>
</tr>
<tr>
<td>Green tea extracts</td>
<td>8</td>
<td>2.8 mg EGCG/kg bw</td>
<td>1.6</td>
<td>0.17 EGCG</td>
<td>1.11</td>
<td>Trace amount</td>
<td>3.4 EGCG</td>
<td>80</td>
</tr>
<tr>
<td>Green tea extracts</td>
<td>8</td>
<td>2.2 mg EGC/kg bw</td>
<td>1.3</td>
<td>0.73 EGC + 5.05 Me EGC</td>
<td>3.09</td>
<td>3.3 EGCG + 12.3 4'Me EGC</td>
<td>1.7 EGC</td>
<td>80</td>
</tr>
<tr>
<td>Green tea extract</td>
<td>4</td>
<td>88 mg EGCG</td>
<td>0.24 EGCG at 1 h</td>
<td>0.46 EG at 1 h</td>
<td>2.0 total catechins</td>
<td>2.0 total catechins</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>Green tea extract</td>
<td>6</td>
<td>109.5, 219, 328 mg EGCG</td>
<td>1.6; 2.4; 2.7</td>
<td>0.21 EC at 1 h</td>
<td>1.96, 4.85, 5.37</td>
<td>5.5, 5.0, 4.9</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Green tea extract</td>
<td>12</td>
<td>0.93 g total catechins</td>
<td>2.3</td>
<td>0.26 EGCG at 3 h</td>
<td>2.02, 8.14, 10.72</td>
<td>2.7, 2.8, 2.5</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Green tea extracts</td>
<td>4</td>
<td>1.64 mg EGC/kg bw</td>
<td>0.5–2</td>
<td>0.8–1.2 EGCG + 3.8–6.9 4'MeE</td>
<td>1.0 EGC; 4.4 'Me EGC</td>
<td>85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green tea</td>
<td>21</td>
<td>640 mg total catechins</td>
<td>1.5</td>
<td>1.8 total catechins</td>
<td></td>
<td></td>
<td>86</td>
<td></td>
</tr>
</tbody>
</table>

Continues
Polymeric proanthocyanidins are not absorbed as such. The detection of proanthocyanidin dimers B1 and B2 in human plasma was reported in only 2 studies (62, 93) (Table 5). The absorption of these dimers was minor, ~100-fold lower than that of the flavanol monomers in the study by Holt et al (62). In vitro and animal studies confirmed that polymerization greatly impairs intestinal absorption (94–96).

However, health effects of proanthocyanidins may not require efficient absorption through the gut. Indeed, these compounds may have direct effects on the intestinal mucosa and protect it against oxidative stress or the actions of carcinogens. In addition, the consumption of proanthocyanidin-rich foods, such as cocoa, red wine, or grape seed extracts, has been shown to increase the plasma antioxidant capacity, to have positive effects on vascular function, and to reduce platelet activity in humans (97). These proanthocyanidin-rich sources always contain 5–25% monomers or other polyphenols, which leaves doubts about whether proanthocyanidins are actually the active compounds in these sources. If they are, then they may have effects through interactions with other components, such as lipids or iron, in the gut.

Biological effects may be attributable not to direct actions of proanthocyanidins themselves but to actions of some of their metabolites that can be more readily absorbed. On the basis of in vitro experiments, Spencer et al (98) suggested that polymers could be degraded into monomers during their transit in the stomach. However, Rios et al (99) clearly demonstrated that this does not occur in humans, probably because the food bolus has a buffering effect, making the acidic conditions milder than required for proanthocyanidin hydrolysis.

Proanthocyanidins are degraded into various aromatic acids by the microflora. The incubation of purified, 14C-labeled, proanthocyanidin oligomers with human colonic microflora led to the formation of m-hydroxyphenylpropionic acid, m-hydroxyphenylacetic acid, and, their p-hydroxy isomers, m-hydroxyphenylvaleric acid, phenylpropionic acid, phenylacetic acid, and benzoic acid (100). Some of these compounds, namely, m-hydroxyphenylpropionic acid and m-hydroxyphenylacetic acid, as well as m-hydroxybenzoic acid, were shown to increase in human urine after consumption of procyanidin-rich chocolate (101). However, the microbial metabolism of proanthocyanidins has never been studied in humans after consumption of purified proanthocyanidin polymers. By feeding rats with purified catechin, dimer B3, trimer C2, or procyanidin polymers, Gonthier et al (102) showed that the extent of degradation into aromatic acids decreased as the degree of polymerization increased; it was 21 times lower for polymers than for the catechin monomer, probably because of the antimicrobial properties and protein-binding capacity frequently described for proanthocyanidins. Therefore, the quantitative importance of the degradation of proanthocyanidins into microbial metabolites must be further evaluated in humans.

### ISOFLAVONES

Isoflavones are provided only by soybean-derived products. They can be present as aglycones or glycosides, depending on the soy preparation. Some authors investigated the differences in bioavailability between aglycones and glycosides by using pure molecules. Contradictory results have been obtained (Table 6). Setchell et al (112) found greater bioavailability of glycosides, as measured from the areas under the plasma concentration-time curves. Izumi et al (110) found greater bioavailability of aglycones, on the basis of Cmax, but they did not measure isoflavone concentrations between 6 and 24 h, whereas Setchell et al (112) reported that the mean time to reach Cmax was prolonged to 9 h after glycoside ingestion. Two other studies found no significant differences in the absorption efficiency for aglycones and glycosides (117, 118).

In contrast, equol production was significantly higher after ingestion of daidzin than after ingestion of daidzein (112, 117). Equol is a bacterial metabolite that has been shown to be more

---

**TABLE 4**

Continued

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of subjects</th>
<th>Dose</th>
<th>Tmax</th>
<th>Plasma concentration</th>
<th>AUC</th>
<th>Urinary excretion</th>
<th>Elimination half-life</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green tea</td>
<td>18</td>
<td>1.04 g total catechins/</td>
<td>0.5–2</td>
<td>1.0 total catechins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>d for 3 d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black tea</td>
<td>12</td>
<td>0.3 g total catechins</td>
<td>2.2</td>
<td>0.17 total catechins</td>
<td>0.53</td>
<td>6.9</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>Black tea + milk</td>
<td>12</td>
<td>0.3 g total catechins</td>
<td>2</td>
<td>0.18 total catechins</td>
<td>0.60</td>
<td>8.6</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>Black tea</td>
<td>15</td>
<td>400 mg total catechins/4 times</td>
<td>0.02 EGC, 0.14 EGC</td>
<td>0.14 ECG, 3.7</td>
<td>88</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black tea</td>
<td>21</td>
<td>140 mg total catechins</td>
<td>1.5</td>
<td>0.34 total catechins</td>
<td></td>
<td></td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Black tea</td>
<td>18</td>
<td>400 mg total catechins/ for 3 d</td>
<td>0.3 total catechins</td>
<td></td>
<td>2.5 EGC, 6.5 EC</td>
<td>86</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Tmax, time to Cmax; AUC, area under the curve; bw, body weight; EC, epicatechin; EGC, epigallocatechin; Me, methyl.

**TABLE 5**

Bioavailability studies of proanthocyanidins or proanthocyanidin-containing foods

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of subjects</th>
<th>Dose</th>
<th>Tmax</th>
<th>Plasma concentration</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocoa beverage</td>
<td>5</td>
<td>256 mg dimers</td>
<td>2</td>
<td>0.041 B2</td>
<td>62</td>
</tr>
<tr>
<td>Grapeseed extract</td>
<td>4</td>
<td>18 mg procyanidin B1</td>
<td></td>
<td>0.011 B1</td>
<td>93</td>
</tr>
</tbody>
</table>

1 Tmax, time to Cmax.
estrogenic than its precursor daidzein in many in vitro studies and in animal models (119). There is great interindividual variability in the capacity to produce equol, and only 30-40% of the Western population are “equol producers.” Equol producers may gain more benefits from soy consumption than do nonproducers (119, 120). Therefore, it would be interesting to find a way to make nonproducers become producers. To date, no clear correlations between dietary habits or microflora composition and the capacity to produce equol have been reported. It would be interesting to separate volunteers into equol producers and nonproducers in future intervention studies designed to investigate the effects of soy isoflavones. Cmax values for equol were measured 12–24 h after isoflavone ingestion (112, 117).

It has long been thought that the greater urinary excretion of daidzein reflects greater bioavailability of this isoflavone, compared with genistein (103). The explanation is that a greater fraction of genistein is eliminated in bile, as observed in rats (121). Plasma kinetic curves often showed a first peak followed by a second, smaller peak. This second peak is likely due to the metabolism of genistein to daidzein and the subsequent production of equol by intestinal bacteria.

### Table 6
Bioavailability studies of isoflavones or isoflavone-containing foods

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of subjects</th>
<th>Dose</th>
<th>Plasma concentration</th>
<th>AUC</th>
<th>Urinary excretion</th>
<th>Elimination half-life</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean milk</td>
<td>12</td>
<td>24.7, 45.9, 70.7 mg Da</td>
<td>0.79, 1.22, 2.24 μmol/L</td>
<td></td>
<td>19.8, 23.7, 20.8 h</td>
<td>5.3, 11.0, 10.0</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19.3, 36.2, 55.7 mg Ge</td>
<td>0.53, 1.10, 2.15 μmol · h/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tofu or texturized vegetable proteins</td>
<td>7</td>
<td>0.34-0.41 mg Da/kg bw</td>
<td>1.44 at 6.5 h</td>
<td></td>
<td>4.9, 0.0</td>
<td></td>
<td>104</td>
</tr>
<tr>
<td>Soybean flour in cow milk</td>
<td>6</td>
<td>0.67 mg Da/kg bw</td>
<td>3.14</td>
<td></td>
<td>13-16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.97 mg Ge/kg bw</td>
<td>4.09</td>
<td></td>
<td>62.0</td>
<td>4.7</td>
<td>105</td>
</tr>
<tr>
<td>Baked soybean powder</td>
<td>7</td>
<td>26.1 mg Da</td>
<td>1.56</td>
<td></td>
<td>35.8 Da + 7 equal</td>
<td>5.8</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30.2 mg Ge</td>
<td>8.0</td>
<td></td>
<td>17.6 Ge</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td>Soymilk</td>
<td>14</td>
<td>0.49 mg Da/kg bw</td>
<td>1.14 at 6 h</td>
<td></td>
<td>48.6</td>
<td></td>
<td>107</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.59 mg Ge/kg bw</td>
<td>1.74 at 6 h</td>
<td></td>
<td>27.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10 mg Gly/kg bw</td>
<td>0.21 at 6 h</td>
<td></td>
<td>55.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soygerm</td>
<td>14</td>
<td>0.55 mg Da/kg bw</td>
<td>1.40 at 6 h</td>
<td></td>
<td>43.8</td>
<td></td>
<td>107</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.15 mg Ge/kg bw</td>
<td>0.49 at 6 h</td>
<td></td>
<td>29.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50 mg Gly/kg bw</td>
<td>0.79 at 6 h</td>
<td></td>
<td>54.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cooked soybeans</td>
<td>10</td>
<td>20 mg Da</td>
<td>3.1</td>
<td></td>
<td>45.0</td>
<td></td>
<td>108</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 mg Ge</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Texturized vegetable protein</td>
<td>10</td>
<td>28 mg Da</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>32 mg Ge</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tofu</td>
<td>5</td>
<td>37 mg Da</td>
<td>1.0</td>
<td></td>
<td>5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>43 mg Ge</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tempeh</td>
<td>4</td>
<td>22 mg Da</td>
<td></td>
<td></td>
<td>38.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 mg Ge</td>
<td></td>
<td></td>
<td>9.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy beverage</td>
<td>12</td>
<td>0.6 mg Da/kg bw</td>
<td>19.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mg Ge/kg bw</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1 mg Gly/kg bw</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean extracts</td>
<td>8</td>
<td>15.7, to 233.7 mg Da</td>
<td>0.77, 16.6</td>
<td></td>
<td></td>
<td></td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13, 210.6 mg Ge</td>
<td>1.04, 21.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20.8, 333.1 mg daidzin</td>
<td>0.17, 3.66</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25.9, 388.8 mg genistin</td>
<td>0.35, 2.56</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy beverage</td>
<td>12</td>
<td>0.6 mg Da/kg bw</td>
<td>2.3</td>
<td></td>
<td></td>
<td></td>
<td>111</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mg Ge/kg bw</td>
<td>8.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure compounds</td>
<td>6</td>
<td>60 mg Da</td>
<td>11.6</td>
<td></td>
<td></td>
<td></td>
<td>112</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60 mg Ge</td>
<td>17.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 mg genistin</td>
<td>16.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 mg genistin</td>
<td>18.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 mg Gly</td>
<td>7.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy isolates</td>
<td>30</td>
<td>0.5–7.8 mg Da/kg bw</td>
<td>6.8</td>
<td></td>
<td></td>
<td></td>
<td>113</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1–16 mg Ge/kg bw</td>
<td>6.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy extract</td>
<td>24</td>
<td>0.28-8.4 mg Da/kg bw</td>
<td>1.7–9.0</td>
<td></td>
<td>14–35 Da</td>
<td>4–18 Ge</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2, 4, 8, 16 mg Ge/kg bw</td>
<td>3.4–25.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy nuts</td>
<td>10</td>
<td>6.6, 13.2, 26.4 mg Da</td>
<td>0.84, 1.05</td>
<td></td>
<td>57.2, 10.1, 18.1</td>
<td>63, 54, 44</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.8, 19.6, 39.2 mg Ge</td>
<td>1.22, 2.21</td>
<td></td>
<td>1:7, 13, 31.2</td>
<td>25.2, 13, 4, 15.8</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0, 4.0, 6.0</td>
<td>4.0, 8.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy isolates</td>
<td>16</td>
<td>0.4, 0.8 mg Da/kg bw</td>
<td>0.55, 0.87</td>
<td></td>
<td>6.7, 9.8</td>
<td>8.9, 8.3</td>
<td>117</td>
</tr>
<tr>
<td>Pure aglycones</td>
<td>15</td>
<td>16 mg Da</td>
<td>0.53</td>
<td></td>
<td>6.2 Da + 7 equal</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.8 mg Ge</td>
<td>0.53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure glycosides</td>
<td>12.5 mg Da eq</td>
<td>4.0</td>
<td>8.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.2 mg Ge eq</td>
<td>5.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Tmax, time to Cmax; AUC, area under the curve; bw, body weight; Da, daidzein; Ge, genistein; Gly, glycitein; eq, equivalents.
\[ \text{\textit{TABLE 7}} \]

Bioavailability studies of hydroxycinnamic acids or hydroxycinnamic acid-containing foods

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of subjects</th>
<th>Dose</th>
<th>( T_{\text{max}} )</th>
<th>Plasma concentration</th>
<th>Urinary excretion</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coffee (200 mL)</td>
<td>10</td>
<td>96 mg chlorogenic acid</td>
<td>1</td>
<td>505 caffeic acid</td>
<td></td>
<td>135</td>
</tr>
<tr>
<td>Red wine (100, 200, 300 mL)</td>
<td>5</td>
<td>0.9–1.8–2.7 mg caffeic acid</td>
<td>1</td>
<td>6.6–18–27</td>
<td></td>
<td>136</td>
</tr>
<tr>
<td>Red wine (200 mL)</td>
<td>10</td>
<td>1.8 mg caffeic acid</td>
<td>0.5–1</td>
<td>37–60</td>
<td></td>
<td>137</td>
</tr>
<tr>
<td>Pure compound</td>
<td>7 ileostomized</td>
<td>1 g chlorogenic acid</td>
<td></td>
<td>0.3</td>
<td></td>
<td>138</td>
</tr>
<tr>
<td>Pure compound</td>
<td>7 ileostomized</td>
<td>500 mg caffeic acid</td>
<td></td>
<td>10.7</td>
<td></td>
<td>138</td>
</tr>
<tr>
<td>Coffee</td>
<td>5</td>
<td>898 mg eq chlorogenic acid/3 times</td>
<td></td>
<td>5.9(^{2})</td>
<td></td>
<td>139</td>
</tr>
<tr>
<td>Artichoke extract</td>
<td>10</td>
<td>124 mg eq chlorogenic acid/3 times</td>
<td>12–43 ferulic acid</td>
<td>5.6(^{2})</td>
<td></td>
<td>140</td>
</tr>
<tr>
<td>Red wine</td>
<td>12</td>
<td>55 ( \mu \text{g} ) caffeic acid/kg bw</td>
<td>2</td>
<td>84</td>
<td></td>
<td>141</td>
</tr>
<tr>
<td>Apple cider (1.1 L)</td>
<td>6</td>
<td>15 mg total hydroxycinnamic acids (&lt;2)</td>
<td></td>
<td>430</td>
<td></td>
<td>35</td>
</tr>
<tr>
<td>Breakfast cereals</td>
<td>6</td>
<td>260 mg ferulic acid</td>
<td>1–3</td>
<td>150–210 ferulic acid</td>
<td>3.1</td>
<td>142</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>5</td>
<td>30 mg ferulic acid</td>
<td></td>
<td>11–25</td>
<td></td>
<td>143</td>
</tr>
<tr>
<td>Beer (4 L)</td>
<td>5</td>
<td>9.4 mg ferulic acid</td>
<td></td>
<td>61.7</td>
<td></td>
<td>144</td>
</tr>
</tbody>
</table>

\(^{1}\) \(T_{\text{max}}\) time to \(C_{\text{max}}\); eq, equivalents; bw, body weight.
\(^{2}\) Ferulic + isofurulic + dihydroferulic + vanillic acids.

\(~3\ h\) later by a second peak, reflecting enterohepatic cycling (112, 117). By using \(^{13}\)C-labeled daidzein and genistein, Setchell et al (116) recently showed that the systemic bioavailability and \(C_{\text{max}}\) were significantly higher for genistein than for daidzein. The limited data for glycitein indicate greater bioavailability than for the other isoflavones (107, 114).

The nature of isoflavone metabolites was the same after glycine or aglycone ingestion. Glycosides are hydrolyzed before absorption and are not recovered as such in plasma (122). Aglycones have been recovered in small proportions, generally \(<5\%\) of the total metabolites (111–113, 123). The main metabolites are \(7\)-\(O\)-glucuronides and \(4'\)-\(O\)-glucuronides, with small proportions of sulfate esters (111, 123, 124). Additional metabolites have been identified in human plasma or urine, including dihydrodaidzein, dihydrogenistein, dihydroequol, \(O\)-desmethylyangolensin, and \(6\)-hydroxy-\(O\)-desmethylyangolensin (125–127).

Elimination of isoflavones is quite slow, with half-life values of \(6–8\ h\) (Table 6). After ingestion of daidzein or genistein at 0.4 or 0.8 mg/kg body weight, baseline concentrations of isoflavones in plasma were regained only after \(~48\ h\) (116). Plasma concentrations should therefore increase with repeated ingestion of soy products. However, Lu et al (128) reported that relative urinary excretion of isoflavones and elimination half-lives progressively decreased during 4 wk of daily soymilk ingestion. Lampe et al (129) did not observe any effect on urinary excretion of 1-mo supplementation with isoflavones.

Another point worth noting is the evidence that high concentrations of isoflavones can be found in breast tissue of premenopausal women and in prostate glands of men (130–132). These are the only available data on polyphenol concentrations in tissues.

**HYDROXYCINNAMIC ACIDS**

Intake of chlorogenic acid varies widely but may be very high, up to 800 mg/d among coffee drinkers (133, 134). Nevertheless, very few studies have addressed the bioavailability of this hydroxycinnamic acid, in comparison with other polyphenols (Table 7).

Olthof et al (138) showed that the esterification of caffeic acid, as in chlorogenic acid, markedly reduced its absorption. This was also observed in rats (145, 146). In fact, the absorption of chlorogenic acid occurs mainly in the colon, after hydrolysis by microbial esterases. It is not clear whether chlorogenic acid is present, as such or in a conjugated form, in human plasma. Nardini et al (135) found only caffeic acid in plasma after the ingestion of coffee. We observed, however, that the preparation of \(\beta\)-glucuronidase from Helix pomatia that is generally used to hydrolyze samples also contains esterases that are able to degrade chlorogenic acid into caffeic acid. Therefore, the possibility that chlorogenic acid is present in plasma but is hydrolyzed during sample treatment cannot be excluded. Intact chlorogenic acid has been detected at low concentrations in nonhydrolyzed urine samples (138, 147). Metabolites other than caffeic acid have been identified after ingestion of chlorogenic acid or caffeic acid, namely, ferulic acid, isofurulic acid, dihydroferulic acid, vanillic acid, 3,4-dihydroxyphenylpropionic acid, 3-hydroxyhippuric acid, and hippuric acid (139, 140, 147). Their quantitative importance remains to be investigated.

Ferulic acid is another abundant hydroxycinnamic acid. When present in free form in tomatoes or beer, it is efficiently absorbed (143, 144). However, ferulic acid is also the main polyphenol present in cereals, in which it is esterified to the arabinoxylans of the grain cell walls. This binding has been reported to hamper the absorption of ferulic acid in rats (148, 149). In humans, Kern et al (142) measured the urinary excretion and plasma concentrations of ferulic acid metabolites after ingestion of breakfast cereals. They deduced from the kinetic data that absorption of ferulic acid from cereals takes place mainly in the small intestine, from the soluble fraction present in cereals. Only a minor amount of ferulic acid linked to arabinoxylans was absorbed after hydrolysis in the large intestine.

**HYDROXYBENZOIC ACIDS**

Very little is known about the absorption and metabolism of hydroxybenzoic acids (150). Their limited distribution in food has resulted in limited interest by nutritionists. However, the few studies addressing the bioavailability of gallic acid in humans revealed that this compound is extremely well absorbed, compared with other polyphenols (Table 8). Plasma concentrations of free and glucuronidated forms of gallic acid and its main
metabolite 4-O-methylgallic acid reached 4 \( \mu \text{mol/L} \) after ingestion of 50 mg pure gallic acid. Such intake is not inconceivable, because red wine usually contains 10–60 mg/L gallic acid. However, gallic acid exists in different forms in fruits, nuts, tea, and red wine, i.e., the free form, esterified to glucose (as in hydrolyzable tannins), or esterified to catechins or proanthocyanidins (92, 154). It would be interesting to compare the bioavailability of the different forms of gallic acid.

**COMPARATIVE BIOAVAILABILITY OF POLYPHENOLS**

Mean values for \( C_{\text{max}} \), time to reach \( C_{\text{max}} \), area under the curve, and relative urinary excretion (related to the ingested dose) were calculated for the different polyphenols (Table 9), on the basis of the data compiled in Tables 1–8. Only data from studies using a single dose of a well-characterized polyphenol source were taken into account. To facilitate comparisons between polyphenols, data were converted to correspond to the same supply of polyphenols, a single 50-mg dose of aglycone equivalent. For this, we assumed that the bioavailability parameters increase linearly with the dose, which has been demonstrated in humans only for EGCG (73). When several doses were investigated in the same study, only a mean value for the whole study was considered.

The most striking result of this survey was that gallic acid is far better absorbed than the other polyphenols. The \( C_{\text{max}} \) values for its metabolites reached 4 \( \mu \text{mol/L} \) with a 50-mg intake, and the relative urinary excretion was 38%. Next are isoflavones, which are the most well-absorbed flavonoids, with \( C_{\text{max}} \) values of \( \sim 2 \mu \text{mol/L} \) after a 50-mg intake and mean relative urinary excretions of 42% for daidzin and 15.6% for genistin. Proanthocyanidins and anthocyanins are very poorly absorbed but, in the case of anthocyanins, all of the metabolites might not have been identified, resulting in underestimation of their bioavailability. Values for catechins are certainly underestimated, because methylated metabolites were not taken into account in some studies. Data are

### Table 9

Compilation of pharmacokinetic data from 97 bioavailability studies

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of subjects</th>
<th>Dose</th>
<th>( T_{\text{max}} )</th>
<th>Plasma concentration</th>
<th>AUC</th>
<th>Urinary excretion</th>
<th>Elimination half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>( h ) ( \mu \text{mol/L} )</td>
<td>( \mu \text{mol h/L} )</td>
<td>% of intake</td>
<td>h</td>
<td></td>
</tr>
<tr>
<td>Pure compound 1</td>
<td>1</td>
<td>50 mg GA</td>
<td>1.8 GA + 2.3 4-MeGA</td>
<td>37.1</td>
<td>5.3 ± 0.8</td>
<td>3.4–8.0</td>
<td></td>
</tr>
<tr>
<td>Pure compound 2</td>
<td>10</td>
<td>50 mg GA</td>
<td>1.8 GA + 2.8 4-MeGA</td>
<td>43.0 ± 6.5</td>
<td>21.4 ± 2.0</td>
<td>7.5–17.4</td>
<td>8.5 ± 0.8</td>
</tr>
<tr>
<td>Assam black tea</td>
<td>10</td>
<td>50 mg GA</td>
<td>2.1 GA + 2.6 4-MeGA</td>
<td>4.5 GA + 9.0 MeGA</td>
<td>39.6</td>
<td>1.1–1.3</td>
<td></td>
</tr>
<tr>
<td>Red wine (300 mL)</td>
<td>2</td>
<td>4 mg GA</td>
<td>0.22 GA + 1.1 4-MeGA</td>
<td>153</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ 0.25 3-MeGA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red wine</td>
<td>12</td>
<td>47 ( \mu \text{g} ) GA/kg bw</td>
<td>0.18 4-MeGA</td>
<td>141</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( T_{\text{max}} \) time to \( C_{\text{max}} \), AUC, area under the curve; GA, gallic acid; MeGA, methylgallic acid.

---

*TABLE 9*

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of subjects</th>
<th>Dose</th>
<th>( C_{\text{max}} )</th>
<th>AUC</th>
<th>Urinary excretion</th>
<th>Elimination half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>( h ) ( \mu \text{mol/L} )</td>
<td>( \mu \text{mol h/L} )</td>
<td>% of intake</td>
<td>h</td>
</tr>
<tr>
<td>Daidzin</td>
<td></td>
<td>6.3 ± 0.6</td>
<td>0.36–3.14</td>
<td>21.4 ± 2.0</td>
<td>21.4–62.0</td>
<td>5.3 ± 0.8</td>
</tr>
<tr>
<td>Daidzein</td>
<td></td>
<td>4.9 ± 1.0</td>
<td>0.9–7.0</td>
<td>12.2 ± 2.0</td>
<td>7.5–17.4</td>
<td>27.5</td>
</tr>
<tr>
<td>Genistin</td>
<td></td>
<td>6.5 ± 0.6</td>
<td>0.46–4.04</td>
<td>23.7 ± 0.7</td>
<td>6.2–45.1</td>
<td>15.6 ± 1.8</td>
</tr>
<tr>
<td>Genistin</td>
<td></td>
<td>4.1 ± 0.6</td>
<td>1.26–4.50</td>
<td>19.8 ± 0.6</td>
<td>10.4–32.2</td>
<td>8.6</td>
</tr>
<tr>
<td>Glycitin</td>
<td></td>
<td>5.0</td>
<td>1.50–2.26</td>
<td>7.9</td>
<td>42.9 ± 12.0</td>
<td>19.0–55.3</td>
</tr>
<tr>
<td>Hesperidin</td>
<td></td>
<td>5.5 ± 0.1</td>
<td>0.21–0.87</td>
<td>2.7 ± 0.7</td>
<td>1.9–4.1</td>
<td>8.6 ± 4.0</td>
</tr>
<tr>
<td>Naringin</td>
<td></td>
<td>5.0 ± 0.2</td>
<td>0.03–1.50</td>
<td>3.7 ± 1.5</td>
<td>0.9–7.0</td>
<td>8.8 ± 3.17</td>
</tr>
<tr>
<td>Quercetin glucosides</td>
<td></td>
<td>1.1 ± 0.3</td>
<td>0.51–3.80</td>
<td>9.8 ± 1.9</td>
<td>5.7–16.0</td>
<td>2.5 ± 1.2</td>
</tr>
<tr>
<td>Rutin</td>
<td></td>
<td>6.5 ± 0.7</td>
<td>0.08–0.52</td>
<td>2.9 ± 0.9</td>
<td>1.6–5.5</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>(Epi)catechin</td>
<td></td>
<td>1.8 ± 0.1</td>
<td>0.09–1.1</td>
<td>1.1 ± 0.3</td>
<td>0.5–2.0</td>
<td>18.5 ± 5.7</td>
</tr>
<tr>
<td>EGCG</td>
<td></td>
<td>1.4 ± 0.1</td>
<td>0.0–1.7</td>
<td>2.0 ± 0.8</td>
<td>1.0–3.6</td>
<td>11.1 ± 3.5</td>
</tr>
<tr>
<td>EGCG</td>
<td></td>
<td>2.3 ± 0.2</td>
<td>0.03–0.38</td>
<td>0.5 ± 0.1</td>
<td>0.2–0.9</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>Gallic acid</td>
<td></td>
<td>1.6 ± 0.2</td>
<td>0.57–4.70</td>
<td>37.7 ± 1.0</td>
<td>36.4–39.6</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td></td>
<td>1.0</td>
<td>0.26</td>
<td>0.3</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>Caffeic acid</td>
<td></td>
<td>1.4 ± 0.6</td>
<td>0.26–1.35</td>
<td>10.7</td>
<td>27.6</td>
<td>3.1–61.7</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td></td>
<td>2.0</td>
<td>0.03</td>
<td>0.4 ± 0.3</td>
<td>0.004–5.1</td>
<td></td>
</tr>
<tr>
<td>Anthocyanins</td>
<td></td>
<td>1.5 ± 0.4</td>
<td>0.2–0.7</td>
<td>0.001–0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proanthocyanidin dimers</td>
<td></td>
<td>2.0</td>
<td>0.02–0.01</td>
<td>0.008–0.03</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( \mu \text{mol/L} \) with a 50-mg intake, and the relative urinary excretion was 38%. Next are isoflavones, which are the most well-absorbed flavonoids, with \( C_{\text{max}} \) values of \( \sim 2 \mu \text{mol/L} \) after a 50-mg intake and mean relative urinary excretions of 42% for daidzin and 15.6% for genistin. Proanthocyanidins and anthocyanins are very poorly absorbed but, in the case of anthocyanins, all of the metabolites might not have been identified, resulting in underestimation of their bioavailability. Values for catechins are certainly underestimated, because methylated metabolites were not taken into account in some studies. Data are

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*Notes:

1. All data were converted to correspond to a supply of 50 mg aglycone equivalent.
2. \( T_{\text{max}} \) time to \( C_{\text{max}} \), AUC, area under the plasma concentration-time curve EGCG, epigallocatechin.*
still scarce for hydroxycinnamic acids, and the calculated mean values are probably not very reliable.

The mean area under the plasma concentration-time curve, \( C_{\text{max}} \) and urinary excretion values clearly show the lower absorption of rutin, compared with quercetin glucosides. Another observation is that galloylation of epigallocatechin markedly reduces its absorption. Gallic acid, quercetin glucosides, catechins, free hydroxycinnamic acids, and anthocyanins, which are absorbed in the small intestine or the stomach, reached \( C_{\text{max}} \) at \( \sim 1.5 \) h, whereas rutin and the flavonanes hesperidin and narin gin, which are absorbed after release of the aglycones by the microflora, reached \( C_{\text{max}} \) at \( \sim 5.5 \) h. The mean time to reach \( C_{\text{max}} \) for chlorogenic acid is surprising, because this compound also must be hydrolyzed by the microflora before absorption. In the sole study considered, however, chlorogenic acid was provided as a liquid (coffee) to fasted volunteers, which might have accelerated the absorption kinetics.

Relative urinary excretion is currently used to estimate the minimal absorption rate but, when polyphenols are highly excreted in bile, as for EGCG and genistein, absorption is underestimated. For most polyphenols, the urinary excretion values were consistent with the plasma kinetic data. Values ranged from 0.3% to 43% of the intake, which demonstrates the great variability in the bioavailability of the different polyphenols.

With respect to the elimination half-lives, it appears that catechins, gallic acid, and flavanones have no chance to accumulate in plasma with repeated ingestion. Some of their metabolites may have longer half-lives, however, and quercetin, with a longer half-life, could accumulate in plasma with repeated ingestion.

Extensive variability was observed among the studies. Tenfold variations in the \( C_{\text{max}} \) values were observed for most compounds. Several factors may explain the variability, such as the food matrix or background diet. Individual variations are also important, and some people might have different levels of metabolizing enzymes or transporters, enabling more efficient absorption of polyphenols.

It is important to realize that the mode of calculation and representation used in this review does not take into account the mean dietary intake of each polyphenol. For example, even if isoflavones are efficiently absorbed, they are usually not the major circulating polyphenols in Western populations, because the isoflavone intake is far lower than 50 mg/d for these populations. In contrast, a single glass of orange juice easily provides 50 mg hesperidin.

This information should be useful for the design and interpretation of intervention studies investigating the health effects of polyphenols.

CONCLUSIONS

Bioavailability varies widely among polyphenols and, for some of compounds, among dietary sources, depending on the forms they contain. The plasma concentrations of total metabolites range from 0 to 4 \( \mu \text{mol/L} \) with an intake of 50 mg aglycone equivalents. The polyphenols that are most well absorbed in humans are isoflavonones and gallic acid, followed by catechins, flavanones, and quercetin glucosides, with different kinetics. The least well-absorbed polyphenols are the proanthocyanidins, the galloylated tea catechins, and the anthocyanins. Data for other polyphenols are still too limited. The plasma kinetics also differ among polyphenol classes, with \( C_{\text{max}} \) being reached after \( \sim 1.5 \) h or \( \sim 5.5 \) h, depending on the site of intestinal absorption.

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Plasma concentrations of the flavonoids hesperetin, naringenin and

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quercetin in human subjects following their habitual diets, and diets
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Hackett AM, Griffiths LA, Broillet A, Wermeille M. The metabolism




Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies

Gary Williamson and Claudine Manach

ABSTRACT
For some classes of dietary polyphenols, there are now sufficient intervention studies to indicate the type and magnitude of effects among humans in vivo, on the basis of short-term changes in biomarkers. Isoflavones (genistein and daidzein, found in soy) have significant effects on bone health among postmenopausal women, together with some weak hormonal effects. Monomeric catechins (found at especially high concentrations in tea) have effects on plasma antioxidant biomarkers and energy metabolism. Procyanidins (oligomeric catechins found at high concentrations in red wine, grapes, cocoa, cranberries, apples, and some supplements such as Pycnogenol) have pronounced effects on the vascular system, including but not limited to plasma antioxidant activity. Quercetin (the main representative of the flavonol class, found at high concentrations in onions, apples, red wine, broccoli, tea, and Ginkgo biloba) influences some carcinogenesis markers and has small effects on plasma antioxidant biomarkers in vivo, although some studies failed to find this effect. Compared with the effects of polyphenols in vitro, the effects in vivo, although significant, are more limited. The reasons for this are 1) lack of validated in vivo biomarkers, especially in the area of carcinogenesis; 2) lack of long-term studies; and 3) lack of understanding or consideration of bioavailability in the in vitro studies, which are subsequently used for the design of in vivo experiments. It is time to rethink the design of in vitro and in vivo studies, so that these issues are carefully considered. The length of human intervention studies should be increased, to more closely reflect the long-term dietary consumption of polyphenols. *Am J Clin Nutr* 2005;81(suppl):243S–55S.

KEY WORDS Polyphenols, flavonoids, procyanidin, bioavailability, isoflavone, quercetin, catechin

INTRODUCTION
It is clear that food components must, by definition, be bioavailable in some form to exert biological effects. There have been major advances in the past few years in our knowledge regarding polyphenol absorption and metabolism (1–4), and it is apparent that most classes of polyphenols are sufficiently absorbed to have the potential to exert biological effects. For example, quercetin after a meal containing onions, catechins after red wine consumption, and isoflavones after soy consumption reach micromolar concentrations in the blood (1, 2, 5–7). These findings demonstrate that polyphenols cross the intestinal barrier and reach concentrations in the bloodstream that have been shown to exert effects in vitro, in some studies.

There are thousands of articles on the effects of polyphenols on biological systems in vitro. However, many of those studies did not take bioavailability and metabolism factors into consideration, and the effects reported in those studies do not necessarily occur in vivo. Although most polyphenols are absorbed to some extent, this is very dependent on the type of polyphenol. The range of concentrations required for an effect in vitro varies from <0.1 μmol/L to >100 μmol/L. Because physiologic concentrations do not exceed 10 μmol/L, the effects of polyphenols in vitro at concentrations of >10 μmol/L are generally not valid, with the possible (but unproven) exception of the intestinal lumen. Furthermore, absorption is accompanied by extensive conjugation and metabolism, and the forms appearing in the blood are usually different from the forms found in food. This indicates that in vitro experiments with the form of polyphenols found in food (the aglycone) are not necessarily relevant to the in vivo situation (8).

There are now intervention studies in the literature, of varying quality, that demonstrate significant biological effects of polyphenol consumption among humans, with the use of many different biomarkers (Tables 1–4). This review examines the effects demonstrated in some of the intervention studies reported in the literature. It considers most of the reports on quercetin, catechins, and procyanidins and some of those on isoflavones. Some of the reports described intervention studies involving consumption of foods and, in many of those cases, it was not proved that the observed effects were attributable to the polyphenol component. This situation may improve in the future, for example, with the use of isogenic lines of onions that differ only in their quercetin contents, allowing comparisons between groups consuming the same food but with different polyphenol contents. The bioavailability issues for each group of polyphenols are discussed in the context of the intervention studies.

HUMAN INTERVENTION STUDIES WITH FLAVONOIDS AND BIOAVAILABILITY ISSUES

Flavonols (including quercetin)
Quercetin is found at high concentrations in onions, apples, tea, broccoli, and red wine and as a component of Ginkgo biloba.

1 From the Nutrient Bioavailability Group, Nestlé Research Center, Lausanne, Switzerland (GW), and the Unité des Maladies Métaboliques et Micronutriments, Institut National de la Recherche Agronomique, Saint-Genès Champanelle, France (CM).
3 Supported by Nestlé (Vevey, Switzerland).
4 Address reprint requests and correspondence to G Williamson, Nutrient Bioavailability Group, Nestle Research Center, Vers chez les Blanc, 1000 Lausanne 26, Switzerland. E-mail: gary.williamson@rdls.nestle.com.
<table>
<thead>
<tr>
<th>Substances given</th>
<th>Principal polyphenol</th>
<th>Dose per day</th>
<th>No. of subjects per group</th>
<th>Biomarkers significantly affected</th>
<th>Biomarkers not significantly affected</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ginkgo biloba</em> extract EGB 761</td>
<td>Quercetin</td>
<td>120 mg extract</td>
<td>90</td>
<td>20</td>
<td>Decrease in blood pressure, increase in fasting plasma insulin and C-peptide</td>
<td>Liver function, coagulation tests, metabolic panel</td>
</tr>
<tr>
<td><em>Ginkgo biloba</em> extract EGB 761</td>
<td>Quercetin</td>
<td>120 mg extract</td>
<td>90</td>
<td>6 hyperinsulinemic subjects</td>
<td>Enhanced hepatic extraction of insulin relative to C-peptide and reduction in plasma insulin</td>
<td>Plasma lipid profiles, blood cell counts</td>
</tr>
<tr>
<td><em>Ginkgo biloba</em> extract EGB 761</td>
<td>Quercetin</td>
<td>160 mg extract</td>
<td>22</td>
<td>22 mountain climbers</td>
<td>Abolition of acute mountain sickness</td>
<td>11</td>
</tr>
<tr>
<td><em>Ginkgo biloba</em> extract EGB 761</td>
<td>Quercetin</td>
<td>320 mg extract</td>
<td>7</td>
<td>5 patients undergoing aortic valve replacement</td>
<td>Decrease in free radicals, delay in myoglobin release</td>
<td>Clinical improvement in outcome, but not significant</td>
</tr>
<tr>
<td><em>Ginkgo biloba</em> extract EGB 761</td>
<td>Quercetin</td>
<td>320 mg extract</td>
<td>1</td>
<td>18 elderly subjects with slight age-related memory impairment</td>
<td>Increase in mental performance</td>
<td>13</td>
</tr>
<tr>
<td>“Phenol-rich diet”</td>
<td>Quercetin, kaempferol</td>
<td>21 mg quercetin, 9 mg kaempferol</td>
<td>6</td>
<td>19</td>
<td>Increase in erythrocyte superoxide dismutase activity, decrease in lymphocyte DNA damage (tail moment)</td>
<td>Plasma α-tocopherol and β-carotene</td>
</tr>
<tr>
<td>Onions</td>
<td>Quercetin</td>
<td>200 g onion</td>
<td>1</td>
<td>6</td>
<td>Increased resistance of lymphocyte DNA to strand breakage, decrease in urinary 8-hydroxy-2'-deoxyguanosine</td>
<td>Urinary malondialdehyde</td>
</tr>
<tr>
<td>Fried onions</td>
<td>Quercetin</td>
<td>50 mg</td>
<td>1</td>
<td>5</td>
<td>6% increase in plasma antioxidant capacity</td>
<td>Susceptibility of LDL to oxidation</td>
</tr>
<tr>
<td>Supplement</td>
<td>Quercetin</td>
<td>250 mg quercetin and other polyphenols</td>
<td>28</td>
<td>27</td>
<td></td>
<td>HDL/LDL cholesterol, platelet aggregation, plasma thromboxane B2, blood pressure, resting heart rate</td>
</tr>
<tr>
<td>Onions</td>
<td>Quercetin</td>
<td>114 mg</td>
<td>14</td>
<td>18</td>
<td></td>
<td>Platelet aggregation, thromboxane B2 production, other hemostatic variables</td>
</tr>
<tr>
<td>Parsley</td>
<td>Apigenin</td>
<td>114 mg</td>
<td>14</td>
<td>18</td>
<td></td>
<td>Platelet aggregation, thromboxane B2 production, other hemostatic variables</td>
</tr>
<tr>
<td>Onion and tea</td>
<td>Quercetin</td>
<td>76–110 mg quercetin and other flavonols</td>
<td>14</td>
<td>10 stable type 2 diabetic patients</td>
<td>Decrease in oxidative damage to lymphocyte DNA</td>
<td>Plasma vitamin C, α-tocopherol, urate, albumin, bilirubin, SOD, GPx, selenium</td>
</tr>
<tr>
<td>Supplement</td>
<td>Quercetin</td>
<td>30 mg</td>
<td>14</td>
<td>4</td>
<td>Decrease in TIMP-1 plasma protein and lymphocyte mRNA</td>
<td>TIMP-2 and matrix metalloprotein-2 lymphocyte mRNA or plasma protein</td>
</tr>
<tr>
<td>Supplement</td>
<td>Quercetin</td>
<td>100 mg [curcumin, 400 mg]</td>
<td>1</td>
<td>15 renal transplant patients</td>
<td>Improved renal function only in patients with elevated serum creatinine, improved urine output and lowered isoprostanes in patients with delayed graft function</td>
<td>Blood pressure, calcineurin levels</td>
</tr>
</tbody>
</table>
Table 1 (Continued)

<table>
<thead>
<tr>
<th>Substances given</th>
<th>Principal polyphenol&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Dose per day</th>
<th>No. of subjects per group&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Biomarkers significantly affected</th>
<th>Biomarkers not significantly affected</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplement</td>
<td>Quercetin</td>
<td>2 × 500 mg</td>
<td>30 men with chronic pelvic pain syndrome</td>
<td>Improvement in NIH prostatis symptom score</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>Supplement</td>
<td>Quercetin</td>
<td>30 mg</td>
<td>14</td>
<td>Improved oxidative resistance of LDL</td>
<td>Plasma triglycerides, total, HDL or LDL cholesterol, vitamin E or C, retinal and carotenoid</td>
<td>23</td>
</tr>
<tr>
<td>Supplement</td>
<td>Quercetin</td>
<td>2 × 500 mg</td>
<td>28</td>
<td>Improvement in cystitis symptoms</td>
<td>No side effects or adverse reactions</td>
<td>24</td>
</tr>
</tbody>
</table>

<sup>1</sup> SOD, superoxide dismutase; GPx, glutathione peroxidase; TIMP, tissue inhibitor of matrix metalloproteinase.

<sup>2</sup> The measured polyphenol; the dose is given in the next column. Ginkgo biloba also contains terpenes, and onions also contain high levels of biologically active sulfur compounds and other nutrients.

<sup>3</sup> Healthy subjects, unless otherwise stated.

Intervention studies with quercetin are shown in Table 1. Some diverse effects have been demonstrated for Ginkgo biloba, but these may also be attributable to the terpenoid component of these extracts. Other studies have shown effects on antioxidant biomarkers, such as increased resistance of lymphocyte DNA to strand breakage, decreased urinary 8-hydroxy-2'-deoxyguanosine concentrations, increased plasma antioxidant capacity, decreased tissue inhibitor of matrix metalloproteinase-1 expression, altered renal function, improved prostatitis symptoms, and improved oxidative resistance to LDL. However, there are also studies that show no effects on these biomarkers.

Despite the lack of convincing evidence for consistent effects of quercetin in vivo in humans, there are numerous studies on the properties of quercetin in vitro. The apparent discrepancy between in vitro and in vivo studies may be partly attributable to absorption and metabolism. Generally, quercetin is not found in the plasma as the free form or as the parent glucoside. At the doses used in the intervention studies noted in Table 1 (21–1000 mg), it would be found exclusively as methyl, sulfate, or glucuronide conjugates (102); when added together, these compounds would represent the equivalent of ∼1–5 μmol/L aglycone equivalents at the highest dose. Lower doses of quercetin are more methylated than higher doses in humans (103). Quercetin has a relatively long plasma half-life of 11–28 h, and a 50-mg dose would lead to concentrations of up to ∼0.75–1.5 μmol/L in plasma (1, 4). There is only limited information on the properties of the conjugates in vitro, although it can be concluded that the conjugates have quantitatively different properties and are generally less biologically active, compared with the aglycones (104, 105). However, deconjugation could occur in some tissues such as the liver (106) or at sites of inflammation (107), leading to reactivation of the conjugated quercetin. The bioavailability issues may partially account for the lack of biological activity in vivo, although the lack of activity may reflect the short-term nature of the studies of quercetin and the selection of inappropriate biomarkers.

Isoflavones (genistein and daidzein)

The intervention studies on isoflavones are the most advanced and sophisticated of those for all of the polyphenols. Studies of the consumption of isoflavones lasting up to 1 year have shown effects on bone biomarkers, such as significant increases in bone mineral density and bone mineral content and changes in bone biomarkers, such as reduced excretion of pyridinium cross-links and increased serum concentrations of bone-specific alkaline phosphatase and osteocalcin. Other effects include changes in LDL and HDL cholesterol concentrations, increases in LDL oxidation lag time, and changes in menopausal symptoms and hot flashes. Many, but not all, of the changes could be related to binding to the estrogen receptor, and this has been reviewed (108, 109).

Isoflavones occur in soy as glycosides, but some fermented products contain free aglycones (110). Consumption of isoflavone-rich foods or of the purified isoflavones themselves leads to appearance in the plasma, with a peak of absorption at 6–8 h (7). A dose of 50 mg of either daidzein or genistein, as typically used in intervention studies (the intervention studies noted in Table 1 used 37–128 mg per person per day), yields a peak plasma concentration of ∼2 μmol/L at ∼6 h (4). The glycosides are not present in plasma, and most of the isoflavones are conjugated as sulfates or glucuronides; some free aglycone is also present, and Setchell et al (7) found 8% of the total daidzein as unconjugated aglycone 2 h after consumption of a dose of 50 mg. This decreased to 3% at steady state, which would apply to the intervention studies, because the studies were conducted for 14–365 d. After 4 wk of 30 mg/d isoflavones, the peak plasma concentrations showed no significant changes at the measurement times of 2 and 4 wk (111), which indicates that the bioavailability of isoflavones does not decrease during long periods of intake. However, we have unpublished data showing that isoflavone bioavailability increases during an extended period of intake (C Manach, unpublished observations, 2004), and this issue is currently unresolved. When administered in intervention studies, the isoflavones are clearly active and affect several biomarkers, especially related to bone. It is not known whether this effect is derived from free aglycone in the plasma, whether the conjugated forms are also active, or whether active deconjugation occurs in the relevant tissues. Some of the isoflavone conjugates are active in vitro (112), although information is very limited.

Catechins [(+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate]

(+)–Catechin and (−)-epicatechin are widely distributed in foods. Catechin concentrations are especially high in broad beans, black grapes, apricots, and strawberries. (−)-Epicatechin is found at high concentrations in apples, blackberries, broad...
### TABLE 2
Human intervention studies of isoflavones or isoflavone-containing foods

<table>
<thead>
<tr>
<th>Substances given</th>
<th>Principal polyphenols</th>
<th>Dose per day of isoflavones</th>
<th>No. of subjects per group</th>
<th>Biomarkers significantly affected</th>
<th>Biomarkers not significantly affected</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy foods</td>
<td>Genistein, daidzein</td>
<td>25–45 mg</td>
<td>6 female subjects (age: 20–29 y)</td>
<td>Follicular phase length increased, peak progesterone concentration delayed, midcycle peaks of luteinizing hormone and follicle-stimulating hormone suppressed, reduction in total cholesterol</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Soy vs placebo</td>
<td>Genistein, daidzein</td>
<td>37 mg</td>
<td>20 postmenopausal women</td>
<td>Bone resorption</td>
<td>Bone stiffness</td>
<td>26</td>
</tr>
<tr>
<td>High, low, and control soy</td>
<td>Genistein, daidzein</td>
<td>73 mg</td>
<td>41 hypercholesteremic men and postmenopausal women</td>
<td>Serum interleukin-6 increased in women</td>
<td>Serum C-reactive protein, serum amyloid A, serum α-tumor necrosis factor</td>
<td>27</td>
</tr>
<tr>
<td>Genistein</td>
<td>Genistein</td>
<td>54 mg</td>
<td>30 women (age: 47–57 y)</td>
<td>~3% increase in bone mineral density in femur and lumbar spine</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>Isoflavone concentrate</td>
<td>Genistein, daidzein</td>
<td>80 mg</td>
<td>29 healthy menopausal women</td>
<td>Endothelial function (flow-mediated dilation)</td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>Isoflavone-rich soy</td>
<td>Genistein, daidzein</td>
<td>80 mg</td>
<td>24 perimenopausal women</td>
<td>Bone mineral density increased 5.6%, bone mineral content increased 10.1%</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Isoflavone extract</td>
<td>Genistein, daidzein</td>
<td>100 mg</td>
<td>40 postmenopausal (2 y) women</td>
<td>Decrease in menopausal symptoms, decrease in total cholesterol and LDL cholesterol</td>
<td>Blood pressure, plasma glucose, HDL or triglyceride levels</td>
<td>31</td>
</tr>
<tr>
<td>Isoflavone-rich soy</td>
<td>Genistein, daidzein</td>
<td>56 mg</td>
<td>24</td>
<td>Plasma 8-epi-prostaglandin F2 decreased by ~20%, LDL oxidation lag time by 10%</td>
<td>LDL-tocopherol, plasma polyunsaturated fatty acids or malondialdehyde</td>
<td>32</td>
</tr>
<tr>
<td>Isoflavone tablet</td>
<td>Genistein, daidzein</td>
<td>80 mg</td>
<td>20 women (age: 50–70 y)</td>
<td>Blood pressure, plasma lipid, plasma lipoprotein, endothelium-(in)dependent dilation</td>
<td></td>
<td>33</td>
</tr>
<tr>
<td>Soy protein foods</td>
<td>Genistein, daidzein</td>
<td>86 mg</td>
<td>31 hyperlipidemic subjects</td>
<td>Decreased circulating oxidized LDL</td>
<td>Plasma lipids</td>
<td>34</td>
</tr>
<tr>
<td>Red clover</td>
<td>Genistein, daidzein, biochanin, formononetin</td>
<td>80 mg</td>
<td>17 women</td>
<td>Increase in arterial compliance by 23%</td>
<td></td>
<td>35</td>
</tr>
<tr>
<td>Soy protein</td>
<td>Isoflavones</td>
<td>90 mg</td>
<td>66 hypercholesterolemic postmenopausal women</td>
<td>Non-HDL cholesterol reduced, HDL cholesterol increased, mononuclear cell LDL receptor mRNA increased, increase in bone mineral content and density of lumbar spine</td>
<td></td>
<td>36</td>
</tr>
<tr>
<td>Soy protein powder</td>
<td>Isoflavones</td>
<td>128 mg</td>
<td>14 premenopausal women</td>
<td>Increase in plasma luteinizing hormone and follicle-stimulating hormone during preovulatory phase, decrease in free T₃ and dehydroepiandrosterone sulfate during early follicular phase, decreased estrone during midfollicular phase</td>
<td>Length of menstrual cycle</td>
<td>37</td>
</tr>
</tbody>
</table>

(Continued)
beans, cherries, black grapes, pears, raspberries, and chocolate. The gallates and the gallocatechins are found almost exclusively in tea, especially green tea. Deducing the intake of catechins during intervention studies is more difficult than for isoflavones, because the non-galloylated forms are widespread and can complicate intake estimates. This can lead to consumption of additional sources of catechins; furthermore, the amounts were not measured in some cases. The situation is clearer for the gallates or galloylated catechins, because they are almost exclusive to green tea. It is difficult to estimate the total flavonoid intake from tea, especially green tea. Deducing the intake of catechins from various intervention studies is more difficult than for isoflavones, because the non-galloylated forms are widespread and can complicate intake estimates. This can lead to consumption of additional sources of catechins; furthermore, the amounts were not measured in some cases. The situation is clearer for the gallates or galloylated catechins, because they are almost exclusive to green tea. It is difficult to estimate the total flavonoid intake from tea, especially green tea. The amounts of catechins administered in various intervention studies were highly variable, and administration was for short periods (1–28 d) (Table 1). With a dose of epigallocatechin gallate (EGCG) of 50 mg, peak plasma concentrations were ~0.15 μmol/L (4). Although the exact percentages vary among individuals, among different studies, and with time after consumption, a substantial amount of the EGCG in plasma is unconjugated. For example, EGCG given to volunteers in one dose of 2 mg/kg body wt yielded 77% of total EGCG as the unconjugated form at 1 h after consumption, with some individuals exhibiting values as high as 100% (113).

For the non-galloylated catechins, doses of 35 or 160 mg (+)-catechin (in red wine or chocolate) yielded plasma concentrations of 0.1–0.2 μmol/L (6, 114), and a similar dose of (−)-epicatechin yielded 0.2 μmol/L in plasma (114, 115). Both (+)-catechin and (−)-epicatechin are present in plasma exclusively as conjugates with methyl, sulfate, or glucuronic acid groups (6, 113, 116). Generally, catechins have short plasma half-lives (2–3 h).

In summary, the intervention studies with monomeric catechins give rise to plasma concentrations on the order of 0.1–0.5 μmol/L, but with rapid clearance. Substantial amounts of unconjugated forms of EGCG would be present in plasma, but all (+)-catechin and (−)-epicatechin is predicted to be conjugated. The percentage of catechins in the plasma that are sulfated or glucuronidated depends on the dose, but this is not usually measured in intervention studies.

**Procyanidins [oligomeric (+)-catechin and (−)-epicatechin]**

Procyanidins are oligomeric catechins, covalently linked together. Dimers and trimers are most common, but the degree of oligomeration can be quite high. Procyanidins are present at particularly high concentrations in cocoa, grapes/wine, and apples and are also found in many fruits, such as blackberries, cherries, figs, and plums (117). Purified procyanidins are weakly bioactive in vitro but exhibit numerous effects in vivo in intervention studies. It is important to note that procyanidins usually occur together with monomeric (+)-catechin and (−)-epicatechin; therefore, it is not clear whether the observed effects are

**Table 2 (Continued)**

<table>
<thead>
<tr>
<th>Substances given</th>
<th>Principal polyphenols¹</th>
<th>Dose per day of isoflavones</th>
<th>Days</th>
<th>No. of subjects per group²</th>
<th>Biomarkers significantly affected</th>
<th>Biomarkers not significantly affected</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>Genistein</td>
<td>54 mg</td>
<td>365</td>
<td>30 women (age: 47–57 y)</td>
<td>Reduced excretion of pyridinium cross-links, increased serum bone-specific alkaline phosphatase and osteocalcin, increase in bone mineral density in femur and lumbar spine</td>
<td>HDL cholesterol, triglycerides, weight, blood pressure, creatinine, steroid hormones</td>
<td>28</td>
</tr>
<tr>
<td>Supplement</td>
<td>Isoflavones</td>
<td>132 mg</td>
<td>84</td>
<td>32 postmenopausal women with diet-controlled type 2 diabetes mellitus</td>
<td>Lower fasting insulin, lower insulin resistance, lower LDL and total cholesterol, lower free thyroxine</td>
<td>No changes in bone mineral density or other bone parameters</td>
<td>38</td>
</tr>
<tr>
<td>Isoflavone-enriched soy protein</td>
<td>Isoflavones</td>
<td>90 mg</td>
<td>365</td>
<td>15 young healthy women</td>
<td>Reduced excretion of bone resorption biomarkers, LDL and serum cholesterol decreased</td>
<td>HDL or VLDL cholesterol, triglycerides, no liver cell damage and no tendency to diabetes</td>
<td>39</td>
</tr>
<tr>
<td>Supplement</td>
<td>Isoflavones</td>
<td>62 mg</td>
<td>28</td>
<td>23 healthy perimenopausal women</td>
<td>Reduced excretion of bone resorption biomarkers, LDL and serum cholesterol decreased</td>
<td>HDL or VLDL cholesterol, triglycerides, no liver cell damage and no tendency to diabetes</td>
<td>40</td>
</tr>
</tbody>
</table>

¹ The measured polyphenol; the dose is given in the next column.
² Healthy subjects, unless otherwise stated.
### TABLE 3
Human intervention studies of monomeric catechins or (+)-catechin-containing foods

<table>
<thead>
<tr>
<th>Substances given</th>
<th>Principal polyphenols</th>
<th>Dose per day</th>
<th>No. of subjects per group</th>
<th>Biomarkers significantly affected</th>
<th>Biomarkers not significantly affected</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black tea, green tea, polyphenol-rich extract</td>
<td>Catechins</td>
<td>900 mL</td>
<td>28</td>
<td>13–16</td>
<td>Serum interleukin-6, interleukin-1β, tumor necrosis factor-α, C-reactive protein, fibrinogen, plasminogen activator inhibitor-1, LDL oxidation, plasma cholesterol or triglycerides, plasma vitamin C or E</td>
<td>41, 42</td>
</tr>
<tr>
<td>Green tea drink</td>
<td>Catechins</td>
<td>5 g of green tea</td>
<td>1</td>
<td></td>
<td>Plasma ascorbic acid increased</td>
<td>43</td>
</tr>
<tr>
<td>Green tea drink</td>
<td>Catechins</td>
<td>150, 300, 450 mL</td>
<td>1 10</td>
<td></td>
<td>Dose-dependent increase in plasma antioxidant activity</td>
<td>44</td>
</tr>
<tr>
<td>Green tea extract</td>
<td>Catechins</td>
<td>254 mg total catechins</td>
<td>1 18</td>
<td></td>
<td>40% decrease in plasma phospholipid hydroperoxide levels</td>
<td>45</td>
</tr>
<tr>
<td>Green tea, black tea, Oolong tea</td>
<td>Catechins</td>
<td>8 cups</td>
<td>3 18</td>
<td></td>
<td>LDL oxidation ex vivo</td>
<td>46</td>
</tr>
<tr>
<td>Green tea extract</td>
<td>Catechins</td>
<td>90 mg EGCG</td>
<td>1 10</td>
<td></td>
<td>Energy expenditure 4% higher, respiratory quotient 3% lower, 24-h urinary noradrenaline 40% higher</td>
<td>48</td>
</tr>
<tr>
<td>Green tea extract</td>
<td>Catechins</td>
<td>0.1 mmol</td>
<td>1 10</td>
<td></td>
<td>Decrease in nonheme iron absorption</td>
<td>49</td>
</tr>
<tr>
<td>Black tea</td>
<td>Catechins</td>
<td>5 cups</td>
<td>28 21</td>
<td></td>
<td>Endothelium-dependent dilation increased by 2.3%, independent dilation by 4.2%, lower soluble P-selectin E-selectin, ICAM-1, VCAM-1, platelet aggregation, coagulation and fibrinolytic factors, F₂-isoprostane excretion</td>
<td>50–52</td>
</tr>
<tr>
<td>Green and black tea</td>
<td>Catechins</td>
<td>1 L</td>
<td>7 13</td>
<td></td>
<td>Trend to increasing ex vivo lag time for lipoprotein oxidation F₂-isoprostane excretion</td>
<td>51, 53</td>
</tr>
<tr>
<td>Green tea extract</td>
<td>Catechins</td>
<td>375 mg catechins (270 mg EGCG)</td>
<td>90 70 Moderately obese patients</td>
<td></td>
<td>Body weight and waist circumference decreased</td>
<td>54</td>
</tr>
<tr>
<td>Green tea extract</td>
<td>Catechins</td>
<td>18 mg</td>
<td>21 8 smokers, 8 nonsmokers</td>
<td></td>
<td>Increased plasma antioxidant capacity Plasma protein oxidation, plasma superoxide dismutase, glutathione peroxidase, catalase, urinary 8-oxododeoxyguanosine</td>
<td>55</td>
</tr>
<tr>
<td>Encapsulated green tea extract</td>
<td>Catechins</td>
<td>3 g extract (=10 cups)</td>
<td>28 20 female subjects on high-linoleic acid diet</td>
<td></td>
<td>Decrease in plasma malondialdehyde Serum lipid, prostaglandins, nitric oxide metabolites, coagulation indicators</td>
<td>56</td>
</tr>
<tr>
<td>Green or black tea, Black tea</td>
<td>Catechins</td>
<td>900 mL tea</td>
<td>28 45</td>
<td></td>
<td>Increase in LDL oxidation lag time ex vivo Serum lipids, LDL oxidation Plasma antioxidant status Total cholesterol, triacylglycerol, apolipoprotein B</td>
<td>57, 58, 59</td>
</tr>
</tbody>
</table>
attributable to the procyanidin component, the monomeric component, or both. The predominant effects are on the vascular system and include substantial increases in plasma antioxidant activity, decreased platelet aggregation (both stimulated and unstimulated), decreased plasma concentrations of lipid peroxide and thiobarbituric acid-reactive substances, decreased LDL cholesterol concentrations, increased HDL cholesterol concentrations, decreased susceptibility of LDL to oxidation, endothelium-dependent blood vessel dilation and decreased blood pressure, beneficial effects on capillary fragility and permeability, increased plasma ascorbate concentrations, decreased P-selectin expression, increased concentrations of nitrosated/nitrosylated species, decreased serum thromboxane concentrations, increased diameters of microvessels, reduced serum thromboxane B2 concentrations, increased plasma homocysteine concentrations, increased plasma vitamin B6 concentrations, maintenance of endothelial function (compared with loss with a high-fat diet), increased platelet-derived nitric oxide production, decreased superoxide release, increased α-tocopherol concentrations, and decreased concentrations of circulating autoantibodies to oxidized LDL (Table 4).

The metabolic fate of procyanidins after consumption is still a mystery. After consumption of 2 g of high-procyanidin grape seed extract by volunteers, the plasma concentrations of procyanidin B1 reached only 10 nmol/L (118); after consumption of 0.375 g cocoa/kg body wt, the plasma concentrations of procyanidin B2 reached only 41 nmol/L (114). When administered in a purified form to rats, procyanidin dimer B3 was not found in the plasma (119). However, human intervention studies with procyanidin-rich foods, as discussed above, show biological effects (Table 4). Either the effects are attributable to currently unidentified metabolites of the procyanidins or the effects are attributable to another component, such as the monomeric catechins (or both).

**Microbial metabolites of polyphenols**

The data on the bioavailability of polyphenols presented above considered only the presence of intact polyphenols in the blood, ie, the ingested compound or its conjugates. The extensive microbiota in the colon also plays a critical role in the metabolism of polyphenols. After microbial enzyme-catalyzed deconjugation of any polyphenol conjugates that reach the colon, there are 2 possible routes available, namely, absorption of the intact polyphenol through the colonic epithelium and passage into the bloodstream (as free or conjugated forms) or breakdown of the original polyphenol structure into metabolites. The absorption data presented above include the contribution of the absorption of intact polyphenols in the colon but do not include the breakdown contribution. Microbial metabolism deserves special consideration, because many of the diverse polyphenols are broken down into simpler phenolic compounds that are common to many different polyphenols. In addition, some of the microbial metabolites could have unique biological effects. For example, the isoflavone daidzein is converted to equol by gut microbiota in ~30–40% of the population, and the equol is absorbed into the bloodstream in these people. There is emerging evidence that “equol producers” demonstrate better effects on some biomarkers, such as bone mineral density, after isoflavone consumption, compared with nonproducers (109). This is an example in which microflora activate a polyphenol to a more potent biologically active compound. Although intervention studies demonstrate an effect for procyanidins, the identity of the active component (or components) is not clear. Although intact procyanidins have some biological effects, they are poorly absorbed in an intact form (114, 118, 119). The active species could therefore be metabolites. Some low-molecular weight metabolites were identified in humans in vivo after consumption of cocoa procyanidins (120), but the biological activities of these metabolites are not known and remain to be investigated.

In summary, there are now many human intervention studies in the literature that show biological effects, but the exact effects depend on the class of polyphenol used. There are clear gaps. Most of the studies were short term, and there is a real need for longer-term studies; very few studies demonstrated a dose-response relationship, and this is also needed for convincing evidence. In addition, most studies, with the exception of those
TABLE 4
Human intervention studies of procyanidins or procyanidin-containing foods

<table>
<thead>
<tr>
<th>Substances given</th>
<th>Principal polyphenols$^1$</th>
<th>Dose per day</th>
<th>Days</th>
<th>No. of subjects per group$^2$</th>
<th>Biomarkers significantly affected</th>
<th>Biomarkers not significantly affected</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semisweet chocolate</td>
<td>Procyanidins, (−)-</td>
<td>420 mg procyanidins, (−)-</td>
<td>1</td>
<td>13</td>
<td>Increase in plasma antioxidant activity, decrease in plasma thiobarbituric acid-reactive substances</td>
<td></td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>epicatechin</td>
<td>epicatechin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chocolate chips</td>
<td>Procyanidins, catechins</td>
<td>220 mg flavonoids</td>
<td>1</td>
<td>18</td>
<td>Reduction in ADP/collagen-stimulated and adrenalin/collagen-stimulated, platelet-related primary hemostasis</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>Cocoa</td>
<td>Procyanidins, catechins</td>
<td>897 mg total</td>
<td>1</td>
<td>16</td>
<td>Inhibition of adrenalin-stimulated platelet activation and function</td>
<td>Thromboxane B2 or 6-keto-prostaglandin, plasma cholesterol, triglycerides</td>
<td>64</td>
</tr>
<tr>
<td>Cocoa powder/dark chocolate</td>
<td>Procyanidins, catechins</td>
<td>466 mg procyanidins</td>
<td>28</td>
<td>23</td>
<td>LDL oxidation lag time decreased by 8%, HDL cholesterol 4% higher</td>
<td>Brachial artery diameter, forearm blood flow, blood pressure, heart rate, plasma nitrite, plasma nitrate</td>
<td>65</td>
</tr>
<tr>
<td>Cocoa</td>
<td>Procyanidins, catechins</td>
<td>70 mg (+)-catechin/ (−)-epicatechin and 106 mg procyanidins (compared with equivalent with low polyphenols)</td>
<td>1</td>
<td>20</td>
<td>Endothelium-dependent dilation of brachial artery from 3.4% to 6.3%, plasma nitrosated/nitrosylated species increased by ~60%</td>
<td>Plasm antioxidant status, plasma oxidation biomarkers</td>
<td>66</td>
</tr>
<tr>
<td>Cocoa tablets</td>
<td>Procyanidins, catechins</td>
<td>234 mg polyphenols</td>
<td>28</td>
<td>32</td>
<td>Increase in plasma ascorbic acid, lower P-selectin expression, lower ADP-induced platelet aggregation, lower collagen-induced platelet aggregation</td>
<td>Plasm antioxidant status, plasma oxidation biomarkers</td>
<td>67</td>
</tr>
<tr>
<td>Cocoa</td>
<td>Procyanidins, catechins</td>
<td>500 mg polyphenols</td>
<td>14</td>
<td>13</td>
<td>Lower systolic and diastolic blood pressure</td>
<td>Heart rate, plasma cholesterol, triglyceride, and glucose concentrations</td>
<td>68</td>
</tr>
<tr>
<td>Chocolate</td>
<td>Procyanidins, catechins</td>
<td>320 mg procyanidin, 104 mg (−)-epicatechin</td>
<td>1</td>
<td>20</td>
<td>Small increase in plasma antioxidant activity</td>
<td>Plasma 8-isoprostane</td>
<td>69</td>
</tr>
<tr>
<td>Cocoa</td>
<td>Procyanidins, catechins</td>
<td>100 g dark chocolate</td>
<td>1</td>
<td>12</td>
<td>Increase in plasma antioxidant activity by 20%</td>
<td>Addition of milk reduced the effect on plasma antioxidant activity</td>
<td>70</td>
</tr>
<tr>
<td>Pycnogenol (French pine bark extract)</td>
<td>Procyanidins, some (+)-catechin</td>
<td>75 mg</td>
<td>30</td>
<td>30 women with melasma</td>
<td>Decrease in average melasma area</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pycnogenol (French pine bark extract)</td>
<td>Procyanidins, some (+)-catechin</td>
<td>150 mg</td>
<td>42</td>
<td>25</td>
<td>Increase of plasma oxygen radical absorbance capacity, decrease in LDL cholesterol, increase in HDL cholesterol</td>
<td>LDL oxidizability, plasma lipid peroxides</td>
<td>71</td>
</tr>
<tr>
<td>Pycnogenol (French pine bark extract)</td>
<td>Procyanidins, some (+)-catechin</td>
<td>200 mg</td>
<td>56</td>
<td>11</td>
<td>Decrease in systolic blood pressure, decrease in serum thromboxane</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>Pycnogenol (French pine bark extract)</td>
<td>Procyanidins, some (+)-catechin</td>
<td>120 mg for 30 d, then 60 mg for 30 d</td>
<td>60</td>
<td>11</td>
<td>Decrease in serum reactive oxygen species, lymphocyte apoptosis and p56$^{14}$, reduction in erythrocyte sedimentation rate</td>
<td></td>
<td>73</td>
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(Continued)
<table>
<thead>
<tr>
<th>Substances given</th>
<th>Principal polyphenols[^1]</th>
<th>Dose per day</th>
<th>Days</th>
<th>No. of subjects per group[^2]</th>
<th>Biomarkers significantly affected</th>
<th>Biomarkers not significantly affected</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pycnogenol (French pine bark extract)</td>
<td>Procyanidins, some (+)catechin</td>
<td>300 mg</td>
<td>60</td>
<td>40 with chronic venous insufficiency</td>
<td>Disappearance of edema and pain, reduction in leg heaviness</td>
<td>Venous blood flow</td>
<td>75</td>
</tr>
<tr>
<td>Pycnogenol (French pine bark extract)</td>
<td>Procyanidins, some (+)catechin</td>
<td>100 mg</td>
<td>1 dose</td>
<td>16 smokers</td>
<td>Decrease in smoking-induced platelet reactivity</td>
<td></td>
<td>76</td>
</tr>
<tr>
<td>Pycnogenol (French pine bark extract)</td>
<td>Procyanidins, some (+)catechin</td>
<td>150 mg</td>
<td>28</td>
<td>60</td>
<td>Decreased platelet aggregation, increased diameter of microvessels</td>
<td>Blood glucose and lipids</td>
<td>77</td>
</tr>
<tr>
<td>Pycnogenol (French pine bark extract)</td>
<td>Procyanidins, some (+)catechin</td>
<td>200 mg</td>
<td>56</td>
<td>19 smokers</td>
<td>Reduced platelet activity, reduced serum thromboxane B2</td>
<td>(No effect in non-smokers)</td>
<td>78</td>
</tr>
<tr>
<td>Pycnogenol (French pine bark extract)</td>
<td>Procyanidins, some (+)catechin</td>
<td>200 mg</td>
<td>30</td>
<td>24 athletes</td>
<td>Increased endurance</td>
<td></td>
<td>79</td>
</tr>
<tr>
<td>Pycnogenol (French pine bark extract)</td>
<td>Procyanidins, some (+)catechin</td>
<td>200 mg</td>
<td>90</td>
<td>4 sub-fertile men</td>
<td>Improved sperm morphologic features</td>
<td>Ham’s F10 capacitated count, motility score</td>
<td>80</td>
</tr>
<tr>
<td>Pycnogenol (French pine bark extract)</td>
<td>Procyanidins, some (+)catechin</td>
<td>1.1, then 1.7 mg/kg body weight</td>
<td>28 + 28</td>
<td>21</td>
<td>Decrease in ultraviolet light sensitivity</td>
<td></td>
<td>81</td>
</tr>
<tr>
<td>Pycnogenol (French pine bark extract)</td>
<td>Procyanidins, some (+)catechin</td>
<td>30 mg</td>
<td>14 + 28</td>
<td>39 patients with endometriosis, severe menstrual pain or chronic pelvic pain</td>
<td>Reduction in menstrual cramps, abdominal pain, and tenderness</td>
<td></td>
<td>82</td>
</tr>
<tr>
<td>Cranberry juice</td>
<td>Procyanidins</td>
<td>50 mL juice</td>
<td>180</td>
<td>150 women with urinary tract infections 9</td>
<td>20% decrease in recurrence of urinary tract infections</td>
<td></td>
<td>83</td>
</tr>
<tr>
<td>Cranberry juice</td>
<td></td>
<td>500 mL juice</td>
<td>1</td>
<td>354 mL juice, 112 men with prostate cancer undergoing radiotherapy</td>
<td>Urinary symptoms</td>
<td></td>
<td>84</td>
</tr>
<tr>
<td>Cranberry juice</td>
<td>Procyanidins</td>
<td>354 mL juice</td>
<td>112</td>
<td>112 men with prostate cancer undergoing radiotherapy</td>
<td>Urinary symptoms</td>
<td></td>
<td>85</td>
</tr>
<tr>
<td>Blueberry</td>
<td>Procyanidins, anthocyanins, quercetin</td>
<td>500 mL juice</td>
<td>1</td>
<td>5</td>
<td>Plasma vitamin C, plasma antioxidant activity</td>
<td></td>
<td>86</td>
</tr>
<tr>
<td>Black currant and apple juice</td>
<td>Procyanidins, anthocyanins, quercetin</td>
<td>1.5 L (9.6 mg quercetin)</td>
<td>5</td>
<td>7</td>
<td>Plasma malondialdehyde decreased, plasma 2-amino-adipic acid semialdehyde increased</td>
<td>Erythrocye 2-aminodiacid semialdehyde, plasma trolox equivalent antioxidant activity, γ-glutamyl semialdehyde</td>
<td>87</td>
</tr>
<tr>
<td>Red wine</td>
<td>Procyanidins, anthocyanins, quercetin</td>
<td>500 mL</td>
<td>1</td>
<td>5</td>
<td>No effect on immune functions (tumor necrosis factor-α, interleukin-2, interleukin-4, lymphocyte proliferation, phagocytic activity)</td>
<td>LDL or HDL cholesterol, plasma triacylglycerol</td>
<td>88</td>
</tr>
<tr>
<td>Red wine</td>
<td>Procyanidins, anthocyanins, quercetin</td>
<td>375 mL</td>
<td>14</td>
<td>9</td>
<td>Decreased plasma lipid peroxides</td>
<td></td>
<td>89</td>
</tr>
<tr>
<td>Red wine</td>
<td>Procyanidins, anthocyanins, quercetin</td>
<td>4 glasses</td>
<td>21</td>
<td>11</td>
<td>Increased plasma homocysteine, increased plasma vitamin B6</td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>Red wine</td>
<td>Procyanidins, anthocyanins, quercetin</td>
<td>240 mL wine</td>
<td>30</td>
<td>6</td>
<td>Maintenance of endothelial function, compared with loss with a high-fat diet</td>
<td></td>
<td>91</td>
</tr>
</tbody>
</table>

[^1]: Procyanidins, some (+)-catechin
[^2]: Some subjects per group are estimated.

(Continued)
with isoflavones, administered food instead of pure compounds, and the effects noted may thus be attributable to some other component in that food. Finally, metabolism by microflora needs to be understood, because the gut microflora probably plays a major role in the biological activity of many polyphenols.

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ABSTRACT
Background: A major portion of the catechins in green tea is not absorbed in the small intestine. Bacteria in the colon convert non-absorbed catechins into simpler phenolic compounds, which may also be absorbed. During the production of black tea, most catechins are polymerized to complex molecules called thearubigins. Little is known about the microbial degradation of these complex polyphenols, but hippuric acid has been identified as a major excretion product associated with black tea consumption.
Objective: To investigate whether green tea and black tea have the same metabolic fate in humans.
Design: Seventeen healthy male volunteers were studied with a randomized, full-crossover design. Each intervention period lasted 4 d, ie, a 2-d run-in period with a low-polyphenol diet followed by a 2-d treatment period. Volunteers consumed a daily dose of 6 g green tea solids, 6 g black tea solids, or 360 mg caffeine. Intervention periods were separated by a 10-d washout period. Twenty-four–hour urine samples were collected during the second day of each treatment period. Hippuric acid was analyzed with HPLC-tandem mass spectrometry.
Results: The mean excretion of urinary hippuric acid during black tea and green tea consumption was 3.75 ± 0.28 mmol/24 h and 0.37 to 0.28 mmol/24 h, respectively (95% CI for the difference: −0.37 to +1.30 mmol/24 h). The hippuric acid excretion during the control treatment was much lower (1.89 ± 0.28 mmol/24 h; P < 0.0001, compared with both black tea and green tea).
Conclusion: The ingestion of either green tea or black tea results in a major increase in the excretion of hippuric acid into urine.

KEY WORDS  
Tea, flavonoid, catechin, theaflavin, thearubigin, metabolism, benzoic acid, hippuric acid

INTRODUCTION

Flavonoids are very common in the human diet, and high concentrations can be found in, for example, red wine, beer, chocolate, and tea. Green and black teas originate from the leaves of Camellia sinensis. The leaves of tea plants contain large amounts (10–25% dry weight) of monomeric flavonoids called catechins. Green tea is made by inactivating the enzymes in the freshly picked leaves. Black tea is produced from fresh green leaves through a process called fermentation. In an enzymatic oxidation, catechins are condensed into theaflavins (dimers) and thearubigins (polymers). Approximately 10% of the flavonoids in black tea are catechins, 10% are theaflavins, and 70% are thearubigins (1, 2). The molecular structure of the thearubigins has not been completely resolved (1, 3).

It has been shown that catechins and theaflavins are absorbed from the intestine (4–7). However, only a small amount of the oral dose can be recovered from urine. The fate of the bulk of the catechins and theaflavins is unknown (8). The bioavailability of the thearubigins has not been studied because of the lack of suitable analytic methods.

Flavonoids that are not absorbed in the small intestine are metabolized by the bacterial flora in the colon (9–11). Experiments with cultured intestinal bacteria have demonstrated that fission of the central C3 ring of catechins is mediated by colonic microorganisms (Figure 1). This type of fission is decisive for the basic structure of the resulting metabolites, ie, hydroxyphenyl-γ-valerolactones and phenolic acids (10). These metabolites are absorbed from the colon, and their urinary concentrations exceed that of the intact flavonoid (12–15).

The microbial degradation of theaflavins and thearubigins has been studied much less, but it is possible that these yield very similar metabolites. Clifford et al (16) used $^1$H nuclear magnetic resonance spectroscopy to analyze the urine of healthy volunteers before and after consumption of black tea. They reported black tea consumption to be associated with a 3-fold increase in the urinary excretion of hippuric acid. Hippuric acid excretion after green tea consumption has not been studied, but hippuric acid may be a major metabolite produced from green tea catechins. The aim of this study was to compare the urinary excretion of hippuric acid after consumption of the same amounts of green tea or black tea.

SUBJECTS AND METHODS

Subjects

The protocol for the study and the information brochure for the healthy volunteers were approved by the Medical Ethical Committee of the Nederlandse Unilever Bedrijven BV. Male nonsmoking volunteers were recruited through written invitations sent to male participants of previous studies. The men had been recruited initially through advertisements in the local newspapers. Inclusion criteria were as follows: age of 18–70 y, body...
mass index of 18–32 kg/m², consumption of alcoholic beverages of < 28 glasses/wk, habitual coffee or tea consumption, < 10 h/wk of intense sporting activities, not on a specific diet, not taking flavonoid-containing supplements, no prescribed medication, and had not participated in a biomedical trial for 3 mo before the start of the study. Of 48 eligible men, 39 apparently healthy volunteers were selected on the basis of these inclusion criteria, a medical history questionnaire, and several routine clinical laboratory values measured in blood, serum, and urine. Finally, 18 men were assigned by lot to participate in the study, and 3 served as backup subjects during the run-in period. The protocol was fully explained to these 21 men, and all gave their written informed consent before the start of the study.

One of the volunteers withdrew from the study during the first run-in period, for personal reasons. He was replaced with the first backup subject. Another participant withdrew from the study during the first intervention, because he judged the taste of the study beverage to be unacceptable. He was not replaced. One subject reported the use of the cholesterol-lowering drug pravastatin (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor) in a routine questionnaire assessment after completion of the first intervention period. He had been taking the drug at a stable dose for several years but had “forgotten” to mention it during the screening visit. No effect of pravastatin on the measures studied was found in the medical literature; therefore, it was decided that the subject would not be excluded from the study.

Seventeen participants completed the study. The characteristics of these 17 male participants were as follows (means ± SD): age, 59.2 ± 11.5 y; height, 1.80 ± 0.09 m; body weight, 80.4 ± 10.4 kg; body mass index, 24.8 ± 2.9 kg/m².

### Experimental design

The subjects were studied with a randomized, full-crossover design with 3 treatments, ie, green tea solids, black tea solids, and caffeine (placebo). Each intervention period lasted 4 d, with a 2-d run-in period with a low-polyphenol diet followed by a 2-d tea treatment period. An overview of the study design is presented in Table 1. Intervention periods were separated by a washout period of almost 10 d, during which the volunteers consumed their habitual diets without any restrictions or additions.

Tea solids were prepared by extracting black or green tea (Lipton Research Blends, Lipton Englewood Cliffs, NJ) with boiling water and spray-drying the resulting extracts. The black and green tea solids were provided as prepacked 1-g portions. Volunteers were instructed to dissolve one portion of tea solids in hot water, to add sugar and milk according to their liking, and to drink the beverage while it was still warm. Volunteers were asked to consume 6 of these 1-g portions per day, at 2–3-h intervals (equivalent to ~12 cups of tea per day).

During the control intervention, a daily dose of 360 mg caffeine (USP quality; Sigma-Aldrich, Zwijndrecht, The Netherlands) was supplemented in 6 gelatin capsules, each containing

### TABLE 1

<table>
<thead>
<tr>
<th>Study design</th>
<th>Sunday</th>
<th>Monday</th>
<th>Tuesday</th>
<th>Wednesday</th>
<th>Thursday</th>
<th>Friday</th>
<th>Saturday</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dietary restrictions</td>
<td>Start, 20:00</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Stop, 07:00</td>
<td>No</td>
</tr>
<tr>
<td>Main hot meal supplied</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Supplement</td>
<td>No</td>
<td>Caffeine</td>
<td>Caffeine</td>
<td>Test article</td>
<td>Test article</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Urine collection</td>
<td>No</td>
<td>No</td>
<td>Start, 07:00</td>
<td>Stop, 07:00</td>
<td>Start, 07:00</td>
<td>Stop, 07:00</td>
<td>No</td>
</tr>
<tr>
<td>Blood collection</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>~16:00</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

The scheme was repeated three times, with 1 wk free of dietary restrictions between the intervention weeks.
60 mg caffeine. This amount of caffeine was equivalent to that in the tea solids. Volunteers were instructed to ingest the capsules at 2–3-h intervals during the day.

For the low-polyphenol diet, volunteers were instructed to refrain from drinking coffee, tea, fruit juices, beer, or wine and from eating onions, kale, broccoli, applesauce, or chocolate during the treatment periods. To compensate for the low caffeine content of the low-polyphenol diet, a daily dose of 360 mg caffeine was supplemented during the run-in days. The main hot meals were provided as commercial frozen meals (IgloMora Group, 's-Hertogenbosch, The Netherlands) with low polyphenol contents. Dietary records were maintained during all 3 intervention periods. Volunteers were asked to replicate the diet that they consumed on the 2 run-in days during the subsequent 2 treatment days. They were also encouraged to repeat their diet from the first intervention during subsequent interventions. A printed copy of their first dietary record was given to them as a reminder.

Urine collection

Twenty-four-hour urine samples were collected both during the second day of the run-in period and during the second day of the treatment period (last day of the intervention). Urine was collected into 500-mL polyethylene flasks containing 3.5 mL of a 50% (by vol) solution of m-phosphoric acid. The pH of 24-h urine samples was adjusted to a value between 2.0 and 3.0 with 50% (by vol) m-phosphoric acid solution. Samples of the acidified urine were stored at −20 °C until analysis, within 2 mo. Creatinine concentrations in urine were analyzed with an Hitachi 912 clinical chemical analyzer (Roche, Almere, The Netherlands).

Analysis of hippuric acid in urine

Because preliminary experiments indicated that the concentrations in some of the samples were below the detection limit of the routine HPLC-ultraviolet method, hippuric acid concentrations in urine were analyzed with HPLC-tandem mass spectrometry. HPLC–electrospray-ionization tandem mass spectrometry was performed by injecting 20 μL of urine that had been diluted 20-fold in mobile phase onto an Xterra C18 column (150 mm × 2.1 mm, 5 μm; Waters, Milford, MA). The isocratic mobile phase consisted of 20 mmol/L ammonium acetate:acetonitrile (92:8, by vol), pH 2.8, at a flow rate of 0.3 mL/min. 4-Aminohippuric acid was used as an internal standard. Detection was performed with a Sciex API 3 Plus triple quadrupole (Sciex/Perkin Elmer/ Applied Biosystems, Gouda, The Netherlands) with turbo-ion spray in positive mode. Tandem mass spectrometry parameters were as follows: source temperature, 500 °C; ion spray, 5500 V; orifice, 37 V; nebulizing gas and curtain gas (both nitrogen) flow rates, 50 and 1.2 L/h, respectively. The mass spectrometry data were collected with multiple-reaction monitoring, with a dwell time of 200 ms, a pause time of 49 ms, and m/z ratios for the parent and daughter ions of 179.9 and 105.1 for hippuric acid and 195.1 and 119.9 for 4-aminohippuric acid (internal standard), respectively.

Calibration curves were obtained with the standard-addition method, because preliminary experiments indicated variable recovery when external calibration was used. Samples were spiked with 0, 0.5, 2.0, 3.5, or 5.0 μmol/L hippuric acid and 50 μmol/L 4-aminohippuric acid. The lower limit of quantification was not assessed, but the sample with the smallest amount produced a signal > 10 times the background noise. Within- and between-assay CVs were 13% and 18%, respectively.

Analysis of catechins in plasma

To check for compliance, blood was collected in EDTA-containing tubes on the second day of each tea treatment, at ~16:00. The concentrations of catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate in plasma were measured essentially as described previously (7).

Analysis of tea samples

Catechins, theaflavins, gallic acid, and caffeine were analyzed with HPLC with diode-array detection. Flavonol-glycosides were converted to the corresponding free aglycones (quercetin, kaempferol, and myricetin) with acid hydrolysis before analysis. Total polyphenol content was analyzed with the Folin-Ciocalteu method (17), with gallic acid as a standard.

Statistical analyses

Statistical analyses were performed with analysis of variance, with treatment as a factor, urinary hippuric acid excretion at the end of the run-in period as a covariable, and subjects and period as blocks. Differences between treatments and 95% CIs were established with Tukey’s test. The SAS version 8.2 software package (SAS Institute, Cary, NC) was used to perform the calculations. Two-sided P values were considered significant at P < 0.05. Urinary hippuric acid excretion and plasma catechin concentrations are reported as least-squares mean ± SEM.

RESULTS

Tea solids

Green tea solids contained 57 mg/g dry weight caffeine, and black tea solids contained 55 mg/g. The total amounts of chemically well characterized polyphenols (ie, catechins, theaflavins, flavonols, and free gallic acid) in the green tea solids and the black tea solids were 0.90 and 0.28 mmol/g, respectively (Table 2). Concentrations of total polyphenols (including thearubigins)
were estimated with the Folin-Ciocalteu assay. Results were 2.21 and 1.51 mmol/g of gallic acid equivalents for the green tea solids and the black tea solids, respectively.

**Hippuric acid in urine**

Mean excretions of urinary hippuric acid were not significantly different during consumption of black tea (3.75 ± 0.28 mmol/24 h) and green tea (4.22 ± 0.28 mmol/24 h; 95% CI of the difference between green tea and black tea: −0.37 to +1.30 mmol/24 h) (Figure 2). Hippuric acid excretion during the control treatment was less than one-half of those values (1.89 ± 0.28 mmol/24 h; P < 0.0001, compared with black tea and compared with green tea). Individual increases in hippuric acid excretion attributable to the consumption of green or black tea (tea control) also were not significantly different (Figure 3). Amounts of hippuric acid excreted during the second day of the run-in period with the low-polyphenol diet (1.87 ± 0.15 mmol/24 h; 51 samples) were not significantly different from the quantities excreted during the control treatment with caffeine, indicating that urinary hippuric acid concentrations had reached low stable values after 35 h with the low-polyphenol diet.

Results were also calculated as millimoles of hippuric acid per mole of creatinine, to correct for small mistakes made during urine collection (2 urine samples missed, 2 samples collected in the wrong container, and 8 mistakes made in the timing of collection were reported). Results were 311 ± 26, 335 ± 26, and 150 ± 26 mmol hippuric acid/mol creatinine during the black tea, green tea, and control supplementation, respectively (P < 0.0001, control compared with black tea and compared with green tea).

The excretion of hippuric acid during the control period varied among individual volunteers by a factor of almost 10 (Figure 2). The responses to the tea treatments also varied extensively, but the consumption of both kinds of tea solids resulted in increases in the urinary excretion of hippuric acid for most volunteers. Hardly any effect of either type of tea on urinary hippuric acid excretion was noticed for 2 volunteers, however, and green tea had a much larger effect than did black tea for 2 other volunteers.

**Catechins in plasma**

Mean plasma catechin concentrations (compliance check) during the control, black tea, and green tea interventions were 20 ± 1, 112 ± 12, and 765 ± 122 mmol/L, respectively. For all volunteers, the lowest plasma catechin concentration was found in the sample collected during the control period and the highest concentration was detected after the green tea intervention, consistent with the much greater amounts of catechins in green tea.

**DISCUSSION**

In this study, the mean urinary excretion of hippuric acid by 17 healthy human volunteers increased by 1.87 and 2.34 mmol/24 h after the consumption of black or green tea solids (6 g/d), respectively. Clifford et al (16) were the first to note the massive increase in urinary hippuric acid excretion after black tea consumption among humans. Among the 9 volunteers they studied, urinary hippurate excretion increased by 1.50 mmol/24 h after the consumption of 8 mugs of black tea per day. The urinary excretion of hippuric acid by the 20 volunteers studied by Olthof et al (14) increased by 1.90 mmol/24 h, on average, after consumption of 4 g of black tea solids per day. The urinary excretion of some phenolic acids [3-hydroxybenzoic acid, 3,4,5-trihydroxybenzoic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylpropionic acid, and 3-(3,4-dihydroxyphenyl)propionic acid] also increased (14); for most of those phenolic acids, however, the increase attributable to the tea supplementation was only on the order of 0.005–0.050 mmol/24 h. The hydroxyphenyl-γ-valerolactones may account for 6–39% of the dose of catechins ingested in green tea.

**FIGURE 2.** Urinary hippuric acid concentrations after treatment with caffeine (control), black tea, or green tea (P < 0.0001, control compared with black tea and compared with green tea; P = 0.1759, green tea compared with black tea).

**FIGURE 3.** Increases in urinary hippuric acid excretion (least-squares mean ± SEM attributable to tea consumption (green tea − control and black tea − control) (open bars), total amounts of simple monomeric and dimeric polyphenols (catechins, theaflavins, flavonols, and gallic acid) consumed per day (black bars), and amounts of total polyphenols consumed per day (hatched bars). Increases in urinary hippuric acid excretion after black and green tea consumption were not significantly different (95% CI for the green tea − black tea difference: −0.36 to +1.30 mmol/24 h; P = 0.1759).
tea (12), but quantitative analysis of these lactones is difficult because of the lack of available standards.

Although all previous results on the bacterial metabolism of tea polyphenols were obtained from experiments with green tea catechins (10), studies reporting massive increases in urinary hippuric acid excretion (14, 16) used a black tea intervention, ie, a mixture of monomeric and polymeric flavonoids. Studies in rats indicated that these animals do not seem to produce hippuric acid from catechin (15), but a species difference between humans and rats cannot be excluded. The volunteers in the present study consumed both green tea and black tea, in a crossover design. Consumption of the 2 types of tea solids resulted in comparable increases in urinary hippuric acid excretion, indicating similar extents of fermentative degradation.

Clifford et al (16) calculated the total amounts of catechins, theaflavins, gallic acid, and flavonols consumed by their volunteers and concluded that these simple polyphenols could not fully account for the increased urinary excretion of hippuric acid. Consequently, they suggested that the complex thearubigins were also converted to hippuric acid. We performed the same calculation and arrived at a similar conclusion; the total amount of simple polyphenols in the daily dose of 6 g of black tea was 1.65 mmol/24 h, compared with a mean increase in hippuric acid excretion during the black tea intervention of 1.87 mmol/24 h. Our results obtained with green tea indicated that only ~45% of the catechins and flavonols were converted to hippuric acid, ie, 5.43 mmol/24 h catechins and flavonols consumed, resulting in additional excretion of 2.34 mmol/24 h hippuric acid. Thearubigins cannot be analyzed because of their complex nature (3). However, the Folin-Ciocalteu assay can be used to measure the total content of polyphenols, including thearubigins, in tea samples relative to a standard such as gallic acid. The daily dose of polyphenols in the black tea solids was 9.08 mmol gallic acid equivalents, and the corresponding increase in hippurate excretion in urine was 1.86 mmol/24 h (20%). For green tea, the corresponding values were 13.3 mmol gallic acid equivalents consumed per day, resulting in an increase in urine hippurate excretion of 2.33 mmol/24 h (18%). Therefore, black tea and green tea consumption had comparable effects on urinary hippuric acid excretion. These results indicate that the lactones and phenolic acids produced from black tea and green tea polyphenols in the human colon are probably quantitatively similar, irrespective of the flavonoid source from which they are derived.

The health benefits of dietary flavonoids have often been attributed to their antioxidant activities, but the microbial metabolites of dietary flavonoids have lower antioxidant activities than do their parent compounds (18). The lower antioxidant activity, however, may be offset by greater bioavailability for these smaller molecules. In addition, dietary flavonoids may have significant effects on the colonic flora (19) and thus confer a type of prebiotic effect. In this respect, it has been noted that not only tea drinking but also wine (15), cider (20), and coffee (14) consumption can result in increases in urinary hippurate acid excretion, indicating that polyphenols from different dietary sources may have similar effects on the colonic flora.

In conclusion, green tea consumption and black tea consumption result in similar amounts of microbial degradation products that are absorbed by the body. These microbial metabolites, and not the native tea flavonoids, may be responsible for at least some of the health effects attributed to tea consumption.

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REFERENCES
Assays for oxidative stress and antioxidant status: applications to research into the biological effectiveness of polyphenols¹–⁴

Andrew R Collins

ABSTRACT
Oxidative stress is a factor in many human diseases, as either cause or effect. A convenient biomarker of oxidative stress is the extent of oxidation of bases in DNA (although measures of lipid or protein oxidation may be equally informative). 8-Oxo-7,8-dihydroguanine or the corresponding nucleoside is most often measured, either chromatographically (gas chromatography-mass spectrometry, HPLC with electrochemical detection, or HPLC-tandem mass spectrometry) or enzymically, with the use of the enzyme formamidopyrimidine DNA glycosylase to convert 8-oxo-7,8-dihydroguanine to DNA breaks, which are detected with alkaline elution, alkaline unwinding, or the comet assay. Estimates of background levels of 8-oxo-7,8-dihydroguanine in normal human cells vary 1000-fold, depending on the technique used. Gas chromatography-mass spectrometry is particularly prone to oxidation of samples during derivatization, whereas HPLC suffers from this artifact to a lesser degree. In a recent interlaboratory study that measured the same samples of human cells, median values obtained with HPLC with electrochemical detection and with formamidopyrimidine DNA glycosylase differed by ~10-fold. There are still questions regarding the actual level of damage, but it is probably approximately one 8-oxo-7,8-dihydroguanine residue per 10⁶ guanines. Assays for antioxidant protection against oxidative damage generally depend on measurements of decreases in a marker of oxidation. Potential dietary antioxidants can be screened with in vitro antioxidant assays or tested in cell culture systems. The best test, however, is in humans. The total antioxidant capacity of plasma is generally insensitive to dietary supplementation with antioxidants or antioxidant-rich foods. An increase in the resistance of lymphocyte DNA to oxidation in vitro is commonly seen, however, and a decrease in endogenous oxidation of DNA may be detected, especially after prolonged supplementation. Am J Clin Nutr 2005;81(suppl):261S–7S.

KEY WORDS Oxidative DNA damage, antioxidant assays, 8-oxoguanine, validation study

INTRODUCTION
Oxidative stress is widely recognized as a factor in many degenerative diseases, as either a cause or effect. The plaques that obstruct arterial flow and cause cardiovascular disease are laid down by macrophages engorged with oxidized LDL (foam cells). Oxidized bases in DNA are potentially mutagenic and so are implicated in the process of carcinogenesis. Diabetes mellitus is associated with oxidative damage to biomolecules, and any inflammatory condition inevitably leads to an increased oxidative burden, because the release of reactive oxygen species by macrophages is part of the body’s defense mechanism.

The role of antioxidants obtained from the diet in protection against disease is a topic of continuing interest and some controversy. Long-term intervention trials with disease as the outcome have yielded generally disappointing results, with increases in rates of lung cancer and cardiovascular disease in 2 large trials in which subjects took a β-carotene supplement (compared with placebo) (1, 2) and no effect in 2 other trials with β-carotene (3) or a mixture of antioxidants (4). An alternative, the molecular epidemiologic approach, has several advantages, notably the small number of subjects needed and the short time scale. Its success depends, however, on the use of reliable validated biomarkers, which are rare. A notable example involves chromosomal aberrations, which were shown in prospective studies to be good indicators of future risk of cancer (5, 6). DNA damage is clearly the ultimate cause of cancer, because DNA base changes can be mutagenic, but, except for some special cases (7), DNA damage is probably better regarded as a marker of exposure to genotoxic agents than as an indicator of the likelihood that cancer will occur in an individual.

In assessments of the effectiveness of particular antioxidants or antioxidant-rich foods, there are several complementary approaches that can be taken, asking the following questions. Does the test substance act as an antioxidant in vitro? Does it protect cells in culture from oxidative damage? When administered to human subjects, does it act as an antioxidant in vivo, decreasing the level of oxidative damage to biomolecules such as lipids, proteins, and DNA? And, under these circumstances, does it render lymphocytes more resistant to challenges with oxidizing agents in vitro?

These experimental approaches depend on the precise measurement of oxidative damage. 8-Oxo-7,8-dihydroguanine in cellular DNA is one of the most commonly measured indices of

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³ Supported by the Scottish Executive Environment and Rural Affairs Department, the United Kingdom Ministry of Agriculture, Fisheries, and Food (later the Food Standards Agency), the International Kiwifruit Organisation, and the European Commission.
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oxidative damage. The serious problem of an oxidation artifact that affects some of the methods used to measure 8-oxo-7,8-dihydroguanine was highlighted by the activities of a consortium of laboratories known as European Standards Committee on Oxidative DNA Damage (ESCODD). It is more than likely that other biomarkers, such as lipid oxidation and protein oxidation, are subject to analogous difficulties. The following account of attempts to optimize the assay of 8-oxo-7,8-dihydroguanine should therefore be seen as a cautionary tale, with implications that reach beyond the estimation of DNA damage.

ASSESSING OXIDATIVE STRESS

Oxidative DNA damage as a biomarker

The popularity of 8-oxo-7,8-dihydroguanine as an indicator of DNA oxidation is probably attributable to the ease with which it can be measured. However, estimates of background levels of 8-oxo-7,8-dihydroguanine in normal human cells can vary by 3 orders of magnitude, depending on the method used. Typically, researchers using gas chromatography-mass spectrometry (GC-MS) have reported amounts of 8-oxo-7,8-dihydroguanine as great as hundreds of residues per 10^6 guanines. HPLC with electrochemical detection (ECD) generally yields lower values, ~5–50 residues per 10^6 guanines. A nonchromatographic approach makes use of the bacterial repair enzyme formamidopyrimidine DNA glycosylase (FPG) to break the DNA at sites of 8-oxo-7,8-dihydroguanine (plus some other oxidized forms of purines). The breaks are then measured with the comet assay, alkaline elution, or alkaline unwinding. This approach typically leads to 8-oxo-7,8-dihydroguanine estimates of ~0.5 residue per 10^6 guanines. The chromatographic methods are prone to oxidation of guanine during isolation and hydrolysis of the DNA, and antioxidants and chelators are used in attempts to control the artifact. The ESCODD was formed to develop reliable methods for measuring 8-oxo-7,8-dihydroguanine, but comparative chromatographic analyses of standard solutions of 8-oxo-7,8-dihydroguanine, calf thymus DNA, oligonucleotides with defined 8-oxo-7,8-dihydroguanine contents, and pig liver have confirmed the intractable nature of the problem of spurious oxidation, with wide variations among participating laboratories (8–11).

Recently, identical samples of HeLa cells were sent to all ESCODD partners, including those using the enzymic methods (9). The median value for 8-oxo-7,8-dihydroguanine measured chromatographically was 5.2 residues per 10^6 guanines, with a range of 1.84-214 residues per 10^6 guanines. The enzymic methods yielded a median value of 0.8 residue per 10^6 guanines (range: 0.06-5.0 residues per 10^6 guanines). To test the accuracy of different methods, we introduced different amounts of additional 8-oxo-7,8-dihydroguanine by treating HeLa cells with a photosensitizer plus visible light. Seven of 8 laboratories using HPLC-ECD were able to detect the dose-response relationship for 8-oxo-7,8-dihydroguanine. Furthermore, when regression lines were calculated, all except one had virtually identical slopes, indicating a high accuracy (Figure 1). GC-MS and HPLC-tandem mass spectrometry methods were unable to detect the dose-response relationship. However, the intercepts of the regression lines with the y-axis, representing background damage, varied over a 75-fold range. It seems that, despite adoption of standard protocols and the use of precautions to prevent adventitious oxidation, this remains a serious problem. The enzymic approach also has some potential problems, ie, the possibility that FPG is detecting lesions other than 8-oxo-7,8-dihydroguanine, leading to overestimation, and the fact that the method relies on indirect calibration. However, the approach does seem to be less prone to adventitious oxidation (12).

In the final stage of ESCODD evaluations (13), lymphocytes were collected from volunteers in the members’ own countries. The median 8-oxo-7,8-dihydroguanine value (based on the means for each set of volunteers) was 4.2 residues per 10^6 guanines with HPLC-ECD, compared with 0.3 residue per 10^6 guanines with enzymic methods. Although there is still a discrepancy between the 2 approaches, it is less than in the past, and there is (at least within the ESCODD) a consensus that 8-oxo-7,8-dihydroguanine levels in many published studies are seriously overestimated.

The conclusions from the ESCODD were as follows. GC-MS is not suitable for measuring basal 8-oxo-7,8-dihydroguanine levels in biological material. HPLC-mass spectrometry has yet to yield convincing results. HPLC-ECD can measure induced damage accurately but still suffers from adventitious oxidation during sample preparation. Enzymic methods are less susceptible to this artifact, but calibration and standardization of methods require attention. The best estimate of background oxidation in normal human cells is 0.3–4 residues of 8-oxo-7,8-dihydroguanine per 10^6 guanines.

Other assays for oxidative stress

For more detailed information, readers are referred to a very thorough critical review of biomarkers of oxidative stress (14). The production of reactive oxygen within cells can be assessed with a fluorimetric technique. The cells are incubated with 2’,7’-dichlorofluorescein diacetate, which they take up; the fluorescence of this compound increases in the presence of superoxide and other oxygen radicals.

Lipid peroxidation can be estimated as the amount of malondialdehyde present in plasma. Malondialdehyde is conjugated to
thiobarbituric acid in the thiobarbituric acid-reactive substances test, and the malondialdehyde-thiobarbituric acid product can be separated from other thiobarbituric acid adducts with HPLC and then measured with fluorescence assessments. However, there are still doubts about the specificity of the assay (additional malondialdehyde can be produced during the reaction with thiobarbituric acid, a situation reminiscent of the problems with 8-oxo-7,8-dihydroguanine), and an additional problem is the instability of malondialdehyde.

As an index of protein oxidation, protein carbonyls can be measured with enzyme-linked immunosorbent assays, Western blotting, or HPLC. The carbonyls are stable, yield quantitative results (allowing detection of dose-response relationships), and appear to reflect disease endpoints in a biologically significant way.

**ASSESSING ANTIOXIDANT ACTIVITY IN VITRO**

**Overview**

The antioxidant value of a particular food component, such as a flavonoid, is not simply a matter of how well it can quench free radicals in a chemically defined reaction system. This is an important factor; but we also need to consider the coexistence of other compounds in the food matrix that may take part in inhibitory, additive, or synergistic interactions, the bioavailability of the compound (how readily is it taken up through the gut and distributed through the body?), the effectiveness of the compound within the cell, and the effectiveness of the compound in the body, both in the short term (after a single dose) and in the long term (when administered on a regular basis).

No single assay can address all of these issues; different approaches are needed. Comparative information can be obtained by simply measuring antioxidant activities of polyphenols (or foods containing them) in vitro but, without data on their bioavailability and metabolism, the relevance of this information to antioxidant effectiveness in organisms is very limited. An understanding of how antioxidants perform within cells is obviously important, although cells in culture are not always reliable models and different cell types in the body can differ widely in their metabolism and consequently in their redox responses. Experiments with human volunteers allow the evaluation of bioavailability and, if good biomarkers are chosen, provide an overview of antioxidant effectiveness.

**Chemical assays for antioxidant activity**

Various assays have been described in which the putative antioxidant is added to a reaction mixture in which free radicals are generated. An example is the 2,2’-azobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay (15), in which ABTS is oxidized by 2,2’-azobis(2-amidopropane). Antioxidants delay the appearance of the colored product of this reaction.

The ferric ion-reducing ability (FRA) assay was devised to assess FRA of plasma (16). It is a colorimetric assay based on the reduction of a colored ferric complex. It has recently found a different application, in the testing of different foodstuffs. A wide range of fruits, vegetables, berries, cereals, and nuts were tested, after a simple standard extraction with methanol/water, and an extremely wide range of values were obtained (17). Some representative FRA values are given in Table 1. The FRA values for extracts from 100 g vary widely, from 0.04 mmol/L for foods such as carrots (generally regarded as being rich in antioxidant carotenoids) to 21 mmol/L for walnuts, 11.3 mmol/L for pomegranates, and 39.5 mmol/L for rosehips (at the top of the list). The FRA value of a food component does not tell us how valuable it would be as an antioxidant within the body, because bioavailability is not addressed, but it does provide useful information on which to base the choice of foods for testing in more elaborate assays.

A variety of phytoestrogens were assessed for antioxidant capacity with 3 tests, ie, the ABTS and FRA assays and electron spin resonance spectroscopy, to determine the ability of the phytoestrogen to quench the stable galvinoxyl radical (18). Results from the electron spin resonance and FRA assays correlated extremely well and showed that most of the phytoestrogens tested had low antioxidant capacity, compared with the standard trolox. The ABTS assay results did not correlate with those for either of the other 2 methods.

We have described a simple in vitro antioxidant assay in which the target molecule for oxidative damage is DNA (19). This is a modified version of the comet assay, in which cells embedded in agarose on a microscope slide are lysed with Triton X-100. The nucleoid DNA present after lysis is particularly sensitive to oxidative attack by H$_2$O$_2$, perhaps because endogenous antioxidants (such as catalase and glutathione) have been leached out of the nucleus, whereas the DNA is still associated with Cu$^{2+}$ ions at the nuclear matrix, which can catalyze production of -OH radicals through the Fenton reaction. Antioxidant activity is assessed as the decreased induction of DNA breaks by H$_2$O$_2$ after pretreatment of nucleoids with the antioxidant. Vitamin C demonstrated concentration-dependent protection against DNA damage. The mixture of antioxidants present in a simple extract of kiwifruit was effective over a wide range of dilutions (Figure 2). This assay has not yet been applied to a variety of different foods; direct comparison with the FRA assay would be a useful exercise.

Lipid oxidation is the basis for another method of assessing antioxidant activity. LDL particles isolated from blood have a certain amount of associated α-tocopherol (vitamin E). LDL particles incubated with macrophages in a suitable medium (containing iron) are initially protected from oxidation by the vitamin E; once this is consumed, however, lipid hydroperoxides accumulate. Alternatively, chemical oxidation of LDL can occur in the presence of Cu(II) alone. If a flavonoid such as quercetin is present in either of these systems, then it delays the appearance of the lipid oxidation products. The flavonoid either scavenges

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**TABLE 1**

FRA of extracts from 100 g of various foods

<table>
<thead>
<tr>
<th>Category</th>
<th>Food</th>
<th>FRA mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cereal</td>
<td>Oat</td>
<td>0.59</td>
</tr>
<tr>
<td>Roots and tubers</td>
<td>Carrot</td>
<td>0.04</td>
</tr>
<tr>
<td>Vegetables</td>
<td>Onion</td>
<td>0.67</td>
</tr>
<tr>
<td>Fruits</td>
<td>Kiwi</td>
<td>0.91</td>
</tr>
<tr>
<td>Berries</td>
<td>Pomegranate</td>
<td>11.3</td>
</tr>
<tr>
<td>Berries</td>
<td>Rose hip</td>
<td>39.5</td>
</tr>
<tr>
<td>Pulses</td>
<td>Raspberry</td>
<td>3.06</td>
</tr>
<tr>
<td>Nuts and seeds</td>
<td>Walnut</td>
<td>21.0</td>
</tr>
</tbody>
</table>

*Data from reference 17.*
free radicals, thus protecting the α-tocopherol and LDL, or acts at a later stage, regenerating α-tocopherol from oxidized α-tocopheroxyl radicals. Red wine, which is rich in polyphenols, inhibited LDL oxidation by macrophages even when diluted 1000-fold (20).

The ability of phytoestrogens to inhibit peroxidation of lipids in microsomes from vitamin E-deficient rats was compared with other antioxidant assays (18). The phytoestrogens were relatively ineffective, compared with vitamin E. This assay and the FRA assay yielded qualitatively similar results.

Testing antioxidant activity in cell cultures

Figure 3 illustrates the usefulness of (and problems associated with) cultured cell model systems for testing food components for both harmful and beneficial effects. The aim was to examine the actions of quercetin, which has been reported to have genotoxic properties, inducing mutations in the Ames test (21); in addition, as a polyphenol, it is known to have antioxidant properties. Quercetin, at a concentration of 25 μmol/L, induces DNA breaks. However, in combination with the DNA-oxidizing agent H₂O₂, the same concentration of quercetin substantially decreases the yield of damage (22). The biological activity of quercetin in the whole body must depend both on the concentration achieved and on the presence of reactive oxygen species.

FIGURE 2. Antioxidant activity of kiwifruit extract analyzed with a modified comet assay. The extract, suitably diluted, was added to the nucleoids in agarose after lysis with Triton X-100, just before addition of H₂O₂ to induce DNA breaks. The level of breakage in nucleoids treated only with H₂O₂ is indicated by the horizontal line, and significant differences from this line (P < 0.05) are indicated with asterisks. Bars indicate SEM. Adapted from reference 19.

FIGURE 3. Effects of quercetin on DNA damage in human lymphocytes. DNA breaks were measured with the comet assay, after 30-min incubation with quercetin at different concentrations (A) or after 30-min incubation with quercetin followed by 5-min incubation with H₂O₂ (B). Bars indicate SEM. Adapted from reference 22.

ANTIOXIDANTS IN HUMANS

Antioxidant capacity of plasma

Various assays for total antioxidant capacity (the total radical-trapping antioxidant potential, trolox equivalent antioxidant capacity, oxygen radical absorbance capacity, and FRA in plasma assays) measure either radical scavenging or reducing capacity. They were devised, or at least have been used, to assess individual antioxidant status, as reflected in body fluids. However, in most human intervention trials in which they have been applied, they have failed to demonstrate an effect of supplementation with antioxidants. This is not at all surprising. The total antioxidant capacity is the sum of many different antioxidants, including dietary ones but also those of endogenous origin. In different assays, albumin and uric acid are major contributors. Supplementation, even if it results in a large increase in the concentration of the particular antioxidant in the plasma, can cause only a modest change in the overall antioxidant capacity.

Antioxidant resistance of lymphocytes and lipids to oxidation

The principle behind this approach is that, if antioxidants are taken up by human subjects as a result of dietary supplementation, then they should reach the bloodstream and enter the blood cells, enhancing the ability of these cells, as well as the plasma lipids, to resist oxidative attack when challenged in vitro with a source of reactive oxygen. The comet assay has been used very successfully to monitor the antioxidant resistance of isolated lymphocytes to DNA damage induced by H₂O₂. After single large doses of vitamin C, vitamin E, or β-carotene (23) or 20-wk supplementation with lower doses of these antioxidants (24), the yield of DNA breaks was significantly decreased. Real foods can also have detectable effects: a 0.5-L drink of kiwifruit juice caused a 25% decrease in breaks at 8 h (19). Supplementation with daily doses of 1 or 2 kiwifruits for 3 wk had a similar effect (25). Porrini and Riso (26) gave female volunteers a daily dose of 25 g of tomato purée (rich in the carotenoid lycopene) for 14 d and observed DNA breaks induced by H₂O₂ decrease by approximately one-half, compared with the level before supplementation. We examined the efficacy of a single “dose” of fried onions,
which are known to be rich in flavonoids. The plasma concentrations of glycosides of quercetin and isorhamnetin were monitored during a 24-h period and reached a peak at 4 h. DNA breaks induced by H$_2$O$_2$ were at their lowest level at 8 h (Figure 4). When onions were combined with tomatoes in the meal, the protective effect of the onions was unexpectedly abolished (27). Very similar results were reported by Lean et al (28), comparing volunteers at intervals after a meal of fried onions. Effects of 2 different concentrations of H$_2$O$_2$, 50 μmol/L (circles) and 200 μmol/L (squares), and the untreated control values, ie, endogenous strand breaks (triangles), are shown. Breaks were measured with the comet assay, and means for the 6 samples are shown. Bars indicate SEM. Adapted from reference 27.

**Endogenous oxidation as a monitor of antioxidant status**

Oxidized bases in lymphocyte DNA are most easily measured with the comet assay, in combination with either FPG, which converts oxidized purines to strand breaks, or endonuclease III, which has the same effect on oxidized pyrimidines. We first demonstrated the effectiveness of an antioxidant mixture (vitamin C, vitamin E, and β-carotene) in decreasing oxidized pyrimidines in lymphocytes from subjects, either smokers or non-smokers, in a 20-wk, placebo-controlled, supplementation trial (24). In another trial, looking specifically for effects of flavonoids, we gave soy milk (1 L/d) to volunteers for 4 wk, with cow’s milk as a control, and found a very substantial decrease in oxidized pyrimidines (31) (Figure 5). At the same time, the phytoestrogens genistein and daidzein were detected in the serum. Licorice extract, containing the flavonoid glycoside glabridin, was similarly protective (30).

Fruits per day, for 3-wk periods) showed highly significant decreases in oxidized bases, although with no sign of a dose-response relationship (25).

It should be noted that, in several studies, the protection of lymphocyte DNA against oxidation in vitro was detected within a few hours after a single dose of antioxidant. In contrast, endogenous oxidation generally requires prolonged supplementation to show an effect. This is not surprising. The endogenous DNA oxidation that we measure is a dynamic steady state, a balance between the input of damage (perhaps modulated by antioxidants) and the removal of damage, through DNA repair. If the input is only a small fraction of the total steady-state level, then it will take some time for a change in input to affect the steady-state level significantly.

The lipid oxidation marker malondialdehyde, measured in plasma, may be as good an index of oxidative stress (or antioxidant protection) in human studies as is DNA damage. Concentrations decreased significantly after supplementation of the diet with vitamin C, vitamin E, β-carotene, and selenium (33). In an attempt to examine the effects of seasonal variations in intake of antioxidant-rich foods, we measured various biomarkers in samples obtained from volunteers in late winter/spring (low consumption of fruits and vegetables) and in late summer/autumn (high consumption). High concentrations of malondialdehyde were found almost exclusively in the winter samples (34) (Figure 6).

**CONCLUSIONS**

Assays for antioxidant status and oxidative damage are many and varied. The simplest ones are purely chemical in vitro reactions or tests in cell cultures. They can yield useful information about mechanisms of action, but extrapolation to effects of dietary antioxidants in vivo is dangerous, because uptake from the gastrointestinal tract and metabolism are not considered.

Supplementation with antioxidants in vivo seems to be the best approach, at least in principle, and experiments should be performed with human subjects if possible. (Animal experiments have not been considered here, but generally the same assays and
approaches as used with human subjects can be applied.) Intervention studies are only as good as the biomarkers used to measure the effects of the intervention. Many published studies use inappropriate methods to measure oxidized bases in DNA, as ESCODD has shown; studies reporting high levels of 8-oxo-7,8-dihydroguanine (≥ 5 residues per 10⁶ guanines) should be re-evaluated. My personal view is that the comet assay, with FPG, is the most convenient and reliable method we have for monitoring levels of 8-oxo-7,8-dihydroguanine and for assessing oxidative stress in general, although of course it should not be used without proper controls and calibration.

Møller and Loft (35) recently published a critical appraisal of human antioxidant supplementation trials that used biomarkers for DNA oxidation (both endogenous damage and resistance to H₂O₂ in vitro). An important criterion was good study design, and many studies were found to be lacking. Those authors concluded that we still do not have a good understanding of the effectiveness of antioxidants in vivo and that in the future we should perhaps concentrate on groups of subjects who are in a state of increased oxidative stress (as a result of disease or smoking, for instance), for whom protective effects of antioxidants may be easier to demonstrate.

If it is important to know about lipids, then measurements of plasma malondialdehyde concentrations or LDL oxidation may yield useful data. If proteins are the main interest, then protein carbonyls can be measured. To date, however, these methods have not been subjected to the rigorous validation checks that have been applied to the markers of DNA oxidation.

Finally, antioxidant activity is not the only biologically important aspect of phytochemicals such as flavonoids. They are also likely to influence xenobiotic-metabolizing enzymes, as well as antioxidant enzymes and DNA repair pathways.

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FIGURE 6. Plasma malondialdehyde concentrations in samples obtained from volunteers in winter and in late summer/autumn in 2 consecutive years, plotted against plasma folate concentrations. Winter samples, triangles (up and down); autumn samples, circles and diamonds. Adapted from reference 34.


Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not?1–4

Barry Halliwell, Joseph Rafter, and Andrew Jenner

ABSTRACT
Foods and beverages rich in phenolic compounds, especially flavonoids, have often been associated with decreased risk of developing several diseases. However, it remains unclear whether this protective effect is attributable to the phenols or to other agents in the diet. Alleged health-promoting effects of flavonoids are usually attributed to their powerful antioxidant activities, but evidence for in vivo antioxidant effects of flavonoids is confusing and equivocal. This may be because maximal plasma concentrations, even after extensive flavonoid intake, may be low (insufficient to exert significant systemic antioxidant effects) and because flavonoid metabolites tend to have decreased antioxidant activity. Reports of substantial increases in plasma total antioxidant activity after flavonoid intake must be interpreted with caution; findings may be attributable to changes in urinary concentrations. However, phenols might exert direct effects within the gastrointestinal tract, because of the high concentrations present. These effects could include binding of prooxidant iron, scavenging of reactive nitrogen, chlorine, and oxygen species, and may inhibit the action of cyclooxygenases and lipoxygenases. Our measurements of flavonoids and other phenols in human fecal water are consistent with this concept. We argue that tocopherols and tocotrienols may also exert direct beneficial effects in the gastrointestinal tract and that their return to the gastrointestinal tract by the liver through the bile may be physiologically advantageous. Am J Clin Nutr 2005;81(suppl):268S–76S.

KEY WORDS Flavonoid, polyphenol, antioxidant, gastrointestinal tract, tocopherol, free radicals, iron, cancer

INTRODUCTION
Foods and beverages rich in flavonoids have been associated with decreased risk of age-related diseases in several epidemiologic studies (1–9), and the concept that flavonoids and other phenolic compounds are responsible is supported by some animal and in vitro studies (10–12). Flavonoids have powerful antioxidant activities in vitro, being able to scavenge a wide range of reactive oxygen, nitrogen, and chlorine species, such as superoxide, hydroxyl radical, peroxyl radicals, hypochlorous acid, and peroxynitrous acid. They can also chelate metal ions, often decreasing metal ion prooxidant activity (13–19). Because considerable evidence indicates that increased oxidative damage is associated with and may contribute to the development of all major age-related diseases (20–26), it has been logical to attribute the alleged protective effects of flavonoids to their antioxidant ability.

There are several caveats, however. First, protective effects of foods and beverages rich in flavonoids do not necessarily equate to protective effects of flavonoids (27, 28). As an analogy, some studies showed that consumption of foods rich in vitamin C decreased levels of oxidative DNA damage in vivo, whereas vitamin C consumption alone did not (reviewed in references 24, 27, and 29). Second, flavonoids and other phenols are complex molecules and are likely to have multiple potential biological activities, such as inhibiting telomerase (30), affecting signal transduction pathways (31–33), inhibiting cyclooxygenases and lipoxygenases (34–36), decreasing xanthine oxidase (37), matrix metalloproteinase (38), angiotensin-converting enzyme (39), and sulfotransferase (40) activities, and interacting with sirtuins (41). Flavonoids may also interact with cellular drug transport systems (42), compete with glucose for transmembrane transport (43), interfere with cyclin-dependent regulation of the cell cycle (44), and affect platelet function (45).

Third, although flavonoids can be absorbed through the gastrointestinal (GI) tract, maximal plasma concentrations achieved are low, usually not more than 1 μmol/L, in part because of rapid metabolism by human tissues and colonic bacteria (46–65). Many of the products of metabolism, such as methylated and glucuronidated forms, must have decreased antioxidant activity because of the blocking of radical-scavenging phenolic hydroxyl groups (60). Therefore, whether plasma concentrations of flavonoids in vivo can be sufficient to exert systemic antioxidant actions (or any of the other effects suggested above) is difficult to predict and must be tested with in vivo experimentation.

Fourth, flavonoids are essentially xenobiotics, as indicated by their patterns of metabolism, and cytotoxic effects have been observed in vitro and in vivo (66–70). Again, the physiologic relevance of such effects is unclear. Many cell culture studies might have been confounded by the rapid oxidation of polyphenolic compounds in cell culture media, generating H2O2 and quinones/semiquinones, which could account for the cellular effects observed (71–73). It is unlikely, however, that all of the cellular effects of flavonoids observed in cell culture studies are...
DO FLAVONOIDS EXERT ANTIOXIDANT EFFECTS IN VIVO?

O’Reilly et al (46) examined this issue among healthy young volunteers who were switched from a flavonoid-rich diet to a flavonoid-poor diet. They measured F2-isoprostane concentrations in plasma, as an index of lipid peroxidation and oxidative DNA damage in white blood cells, with gas chromatography-mass spectrometry (46, 76). The experiment was a randomized crossover study with two 14-d treatments with a flavonoid-poor diet or a flavonoid-rich diet, with a 14-d washout period between treatments. During the flavonoid-rich dietary treatment period, subjects were asked to consume one 150-g onion cake (containing 89.7 mg quercetin) and one 300-mL cup of black tea daily. During the flavonoid-poor dietary treatment period, subjects were asked to avoid the consumption of specified flavonoid-rich foods and of tea and to consume 6 g/d high-oleic acid sunflower oil (containing 76% 18:1 and 14% 18:2n-6), as contained in the 150-g onion cake. Subjects were advised to make no changes to their diets or lifestyle other than those necessary for compliance with the study. During the last 7 days of each dietary treatment phase, subjects were asked to maintain a 7-d food diary. At the end of each treatment phase, venous blood samples were collected from subjects after an overnight fast, and height and weight were recorded. With the flavonoid-rich diet, plasma quercetin concentrations increased from undetectable concentrations to 221.6 ± 37.4 nmol/L (n = 32).

F2-isoprostane measurement is currently regarded as the best method to measure lipid peroxidation in vivo (77, 78). Concentrations remained unchanged whether the subjects were on the flavonoid-rich diet or the flavonoid-poor diet. There was also no effect on the plasma concentrations of oxidized LDL, measured as malondialdehyde-LDL antibody titers (46). There is controversy regarding how best to measure oxidative DNA damage; therefore, it is best not to accept conclusions regarding the effects of dietary interventions on this parameter unless they are supported by several studies using different methods (24). Nevertheless, our studies provided no evidence for an antioxidant effect of quercetin in vivo, among the healthy subjects examined, against either lipid oxidation or oxidative DNA damage (46, 76).

How do our data compare with those of others? The findings are mixed but, overall, the results are consistent with our conclusions. We confine our comments to recent studies with humans, and we avoid discussing studies carried out with unreliable biomarkers, such as plasma thiobarbituric acid-reactive substances. Boyle et al (79) found that rutin supplementation did not affect urinary concentrations of 8-hydroxy-2′-deoxyguanosine (8OHdG) (a putative biomarker of whole-body oxidative DNA damage) (80), F2-isoprostanes, or malondialdehyde in human volunteers, but there was an effect on pyrimidine oxidation products, as measured with the comet assay (although not on endogenous DNA strand breaks) (79). Fruit juice consumption decreased oxidative DNA damage in lymphocytes in one study (81) and plasma F2-isoprostane concentrations in others (82, 83), but fruits and vegetables failed to decrease any marker of oxidative damage, including concentrations of F2-isoprostanes and DNA damage markers, in other studies (84, 85). Grape skin extract (86) and parsley (87) were reported not to decrease concentrations of end products of oxidative protein damage, measured as plasma protein 2-aminoacidic semialdehyde residues, in healthy volunteers. Caccetta et al (88) found that plasma F2-isoprostane concentrations decreased significantly among human smokers after consumption of alcohol-free red wine, but either red or white wine alone had no effect, which perhaps suggests a prooxidant action of other wine constituents, such as alcohol. Kiwi fruit consumption decreased DNA base oxidation, as measured with the comet assay, among human volunteers, possibly by accelerating DNA repair (89), and Thompson et al (90) reported decreased lipid peroxidation (urinary isoprostanes and malondialdehyde) and lymphocyte 8OHdG concentrations among subjects who consumed more fruits and vegetables, which contradicts other studies (84, 85). Similarly, green tea extract failed to decrease urinary isoprostane concentrations among healthy female subjects (91), as did either green or black tea in a study with mainly male subjects (92), but there was a decrease in plasma concentrations of phosphatidylcholine hydroperoxide, an acceptable biomarker of lipid peroxidation, in a study with male subjects in Japan (93). In other studies, green tea was observed to decrease urinary 8OHdG concentrations and lipid peroxidation (measured as malondialdehyde concentrations in the urine) among both smokers and nonsmokers (94, 95). However, black tea had no effect in one of those studies (95). It should be noted that concentrations of malondialdehyde in the urine, unlike those of isoprostanes, can be affected by changes in diet (96–99); therefore, use of urinary malondialdehyde concentrations as a measure of lipid peroxidation must be undertaken with caution if the diet is changed. Young et al (100), in a well-designed, crossover, intervention study with subjects on a low-flavonoid diet, found no effect of green tea extract on urinary 8OHdG excretion. Interestingly, they also found that the low-flavonoid diet itself (excluding tea, wine, fruits, and vegetables) decreased plasma protein oxidation (2-aminoacidic semialdehyde and γ-glutamyl semialdehyde) and urinary 8OHdG excretion, in apparent contradiction to other studies (as reviewed above; also see reference 101). In another study (102), those authors noted increased plasma protein oxidation after fruit juice intake. Much more work must be devoted to the establishment of generally acceptable biomarkers of oxidative protein damage; even F2-isoprostanes and 8OHdG do not fulfill all of the criteria for ideal biomarkers (25, 103). Finally, diets enriched in soy were found to decrease plasma F2-isoprostane concentrations among human volunteers (104). It is clear that the data are confusing and self-contradictory.

INTERPRETATION OF CHANGES IN LDL OXIDIZABILITY AND PLASMA TOTAL ANTIOXIDANT CAPACITY

Several other biomarkers are frequently used to assess in vivo antioxidant effects of phenols. For example, many studies have examined the effects of flavonoids on the resistance of LDL to ex vivo oxidation. However, such studies are difficult to interpret, because flavonoids and their metabolites that might partition between lipoproteins and plasma in the circulation could conceivably wash out from LDL during the lipoprotein isolation procedures, which are usually prolonged. Measurement of
changes in the lag time to LDL oxidation ex vivo must be performed with great care, to avoid misinterpretation (eg, attributable to seasonal effects among humans) (105, 106). Therefore, we place little weight on such studies. Similarly, several reports claimed changes in plasma total antioxidant capacity after consumption of phenolic compounds (107); this is worth some thought. Plasma total antioxidant capacity, as measured with a range of assays, is $> 10^3 \mu mol/L$ (22). Detection of a statistically significant increase in most assays would thus require a minimum of 20–50 $\mu mol/L$ extra antioxidant to be present. However, concentrations of unconjugated flavonoids found in vivo, even with high dietary intakes, are far below this, usually $\leq 1 \mu mol/L$ (see above). Some metabolites might exert significant antioxidant activity but this seems unlikely, because modifications of hydroxyl groups decrease antioxidant ability and concentrations of metabolites are quite low. It is more likely that the interventions cause increases in the concentrations of the major plasma antioxidants, such as ascorbate and urate, and increases in urate concentrations are not necessarily beneficial (108). Finally, it must be emphasized that any effect on any measurable parameter observed with fruit juices, beverages, soy products, or vegetables is not necessarily an effect of the flavonoids or other phenolic compounds that the products contain.

We conclude that the available literature provides no consistent support for systemic antioxidant effects of dietary phenolic compounds. In addition, alterations in the concentrations of even generally accepted biomarkers could be attributable to accelerated removal (eg, DNA repair or metabolism of F2-isoprostanes), generally accepted biomarkers could be attributable to accelerated removal (eg, DNA repair or metabolism of F2-isoprostanes), rather than decreased formation, and decreases in such concentrations should not automatically be assumed to represent antioxidant effects (103). It is also not clear whether the effects of flavonoids on cyclooxygenase/lipoxygenase observed in vitro (109).

DO FLAVONOIDS ACT IN THE GI TRACT?

We proposed that antioxidant and other protective effects of flavonoids and other phenolic compounds could occur before absorption, within the GI tract itself (75). This could account for the ability of flavonoid-rich foods to protect against gastric, and possibly colonic, cancer, although it must not be assumed that any protective effect of flavonoid-rich foods is attributable to antioxidant actions of the flavonoids (110) or to flavonoids at all, rather than to other components in the foods. For example, ingestion of green tea was reported to rapidly decrease prostaglandin E2 concentrations in human rectal mucosa (111), consistent with inhibition of cyclooxygenase activity (34).

The logic behind our hypothesis (75) is that phenolic compounds present in plasma at $< 1 \mu mol/L$ concentrations are present in the stomach and intestinal lumen at much higher concentrations after consumption of foods and beverages rich in such compounds (112–115). Because absorption of phenolic compounds is incomplete, they enter the colon, where they and their products of bacterial fermentation can exert beneficial effects. Indeed, high-flavonoid diets probably influence the microbial composition of gut flora (113, 114). This concept led us to perform studies measuring the phenolic content of human fecal material, as described below.

The GI tract is constantly exposed to reactive oxygen, chlorine, and nitrogen species, many from the diet and others from activation of phagocytes in the gut. The stomach is especially affected; indeed, Kanner and Lapidot (116) referred to the stomach as a “bioreactor.” Sources of reactive species include the following: 1) mixtures of ascorbate and Fe$^{2+}$ in the stomach during iron uptake, which represent a powerful prooxidant combination (22); 2) heme proteins in the diet, which are also potential powerful prooxidants (22); 3) lipid peroxides, cytoxic aldehydes, and isoprostanes in the diet (96, 98, 116–118) [gastric juice may promote lipid peroxidation (116)]; 4) nitrite in saliva and in foods converted to HNO$\_2$ by gastric acid, forming nitrosoating and DNA-deaminitizing species (75, 119); 5) high concentrations of H$_2$O$_2$ in certain beverages (75, 103, 120, 121); 6) the presence in the GI tract of highly oxidizable, prooxidant, phenolic compounds such as hydroxyhydroquinone (103, 122); and 7) activation of immune cells naturally present in the GI tract by diet-derived bacteria and toxins (123).

Flavonoids and other phenolic compounds might exert direct protective effects in the GI tract, by scavenging reactive oxygen and chlorine species. They could inhibit heme protein-induced peroxidation in the stomach. They are able to inhibit DNA base deamination by HNO$\_2$-derived reactive nitrogen species (119). Phenols might up-regulate toxin-metabolizing or antioxidant defense enzymes in the GI tract (124, 125). They might chelate redox-active transition metal ions and decrease their prooxidant potential (17, 22). Dietary iron is usually not completely absorbed, especially among subjects on Western diets. Unabsorbed dietary iron enters the feces, where it could represent a prooxidant challenge to the colon and rectum (126–130). Indeed, diets rich in fat and low in fiber may aggravate this prooxidant effect (123). Phenolic compounds, by chelating iron, may help to alleviate prooxidant actions of colonic iron (Figure 1). Effects of ascorbate and vitamin E in decreasing fecal mutagenicity have been reported (131).

WHAT PHENOLIC COMPOUNDS ARE PRESENT IN THE GI TRACT?

The phenolic compounds in the GI tract include unabsorbed compounds from the diet plus products of microbial metabolism by the gut flora; considerable evidence shows that this metabolism is extensive, and diets rich in phenols probably have effects on the composition of the colonic flora (113, 114, 132–138). We therefore examined the content of phenolic compounds in the human colon. In recent years, many studies have shown that components of the aqueous phase of human feces (fecal water) are more efficient in altering the growth characteristics of colonocytes than are components of the solid phase (139–142). Because fecal water interacts more with the colonic epithelium than does the solid fecal phase and thus may have more influence on the development of colon disease, we measured the concentrations of phenolic compounds in human fecal water. This aqueous fraction contributes an average of 70–75% of total fecal wet weight.

Fecal water was prepared as described previously (139). Briefly, stool samples were homogenized in a stomacher (2 min) and centrifuged at $30,000 \times g$ for 2 h, and the upper water layer was filtered. Samples were acidified and loaded onto solid-phase
extraction columns containing diatomaceous earth (100 mg/100 μL fecal water sample). After 5 min, phenolic compounds were eluted with 1.8 mL ethyl acetate. The organic solvent was removed with nitrogen gas, and the dry sample was derivatized with 10 μL acetonitrile plus 50 μL N,O-bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane for 4 h at 50 °C. Derivatized samples were analyzed with gas chromatography–mass spectrometry, with helium as the carrier gas and with a fused silica capillary column (12 m × 0.2 mm inside diameter) coated with cross-linked 5% phenylmethylsiloxane (film thickness: 0.33 μm; Agilent/J & W, Palo Alto, CA). Selected-ion monitoring was performed with the electron-ionization mode, at 70 eV.

Concentrations of phenolic compounds were highly variable among individuals and, for each individual, were affected by diet (data not shown). Quercetin, naringenin, isorhamnetin, formononetin, and hesperetin were the major flavonoid components. All other polyphenolic compounds were present at < 0.2 μmol/L (Table 1). In contrast, phenolic compounds of lower molecular mass, some but not all (143, 144) of which are likely to be products of microbial degradation, were present at much higher concentrations (Table 2) than were flavonoids. Major components were phenylacetic acid, 3-phenylpropionic acid, 3,4-dihydroxycinnamic acid (caffeic acid), 3-hydroxyphenylacetic acid, benzoic acid, 3-(4-hydroxy)phenylpropionic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxy-3-methoxyphenylcinnamic acid (ferulic acid), and 3,4-dihydroxyphenylpropionic acid. Concentrations of other phenolic acids and phenolic compounds ranged from 0.04 to 8.5 μmol/L (mean concentrations). Although the relative contributions of different sources of phenolic acids in the colon, including diet, microbial metabolism, and excretion from colonic cells into the GI tract, has yet to be established, we are currently investigating the concentrations of phenolic compounds in the fecal bulk, to evaluate the bioavailability of fecal phenolic compounds in the colon.

### TABLE 1

<table>
<thead>
<tr>
<th>Polyphenol</th>
<th>Mean concentration (μmol/L)</th>
<th>Lowest concentration observed (μmol/L)</th>
<th>Highest concentration observed (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>0.74</td>
<td>0.00</td>
<td>2.31</td>
</tr>
<tr>
<td>Naringenin</td>
<td>0.61</td>
<td>0.00</td>
<td>4.04</td>
</tr>
<tr>
<td>Isorhamnetin</td>
<td>0.51</td>
<td>0.00</td>
<td>4.44</td>
</tr>
<tr>
<td>Formononetin</td>
<td>0.36</td>
<td>0.00</td>
<td>3.20</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>0.23</td>
<td>0.00</td>
<td>1.65</td>
</tr>
<tr>
<td>Daidzin</td>
<td>0.10</td>
<td>0.00</td>
<td>0.61</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>0.09</td>
<td>0.01</td>
<td>0.31</td>
</tr>
<tr>
<td>Apigenin</td>
<td>0.07</td>
<td>0.00</td>
<td>0.31</td>
</tr>
<tr>
<td>Catechin</td>
<td>0.06</td>
<td>0.00</td>
<td>0.31</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.05</td>
<td>0.00</td>
<td>0.16</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>0.04</td>
<td>0.00</td>
<td>0.28</td>
</tr>
<tr>
<td>Eriodictyol</td>
<td>0.04</td>
<td>0.00</td>
<td>0.27</td>
</tr>
<tr>
<td>Diosmetin</td>
<td>0.03</td>
<td>0.00</td>
<td>0.20</td>
</tr>
<tr>
<td>Phloretin</td>
<td>0.03</td>
<td>0.00</td>
<td>0.07</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>0.02</td>
<td>0.00</td>
<td>0.12</td>
</tr>
<tr>
<td>Genistein</td>
<td>0.01</td>
<td>0.00</td>
<td>0.05</td>
</tr>
<tr>
<td>Diosmetin</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Luteolin</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Derivatized samples were injected into an Agilent gas chromatography–mass spectrometry system and analyzed with selected-ion monitoring. Concentrations were calculated with a standard calibration curve obtained with pure phenolic standards analyzed under identical conditions. ND, not detected.
Vitamin E comprises multiple stereoisomers of 4 tocopherols (α, β, γ, and δ) and 4 tocotrienols (22, 145). All appear to be absorbed from the GI tract, but a tocopherol transfer protein in the liver selects α-tocopherol for incorporation into plasma lipoproteins, leading to ejection of some of the other tocopherols into the bile and thus back into the GI tract (145). γ-Tocopherol is also rapidly catabolized (146). High concentrations of these vitamin E constituents can be present in the fecal matter, relatively much higher than those in plasma (Table 3), especially for δ-tocopherol, γ-tocopherol, and the tocotrienols. It is possible that these agents, like the flavonoids, exert beneficial effects in the GI tract. Even α-tocopherol may not be completely absorbed (147), especially if

<table>
<thead>
<tr>
<th>Phenolic compound</th>
<th>Mean concentration (µmol/L)</th>
<th>Variance (SD)</th>
<th>Lowest concentration observed (µmol/L)</th>
<th>Highest concentration observed (µmol/L)</th>
</tr>
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<tbody>
<tr>
<td>Phenylacetic acid</td>
<td>409.55</td>
<td>87</td>
<td>8.43</td>
<td>1236.24</td>
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<tr>
<td>3-Phenylpropionic acid</td>
<td>287.05</td>
<td>82</td>
<td>8.71</td>
<td>657.37</td>
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<tr>
<td>3,4-DiOH-cinnamic acid (caffeic acid)</td>
<td>136.05</td>
<td>138</td>
<td>6.63</td>
<td>670.77</td>
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<td>3-OH-phenylacetic acid</td>
<td>56.65</td>
<td>137</td>
<td>0.23</td>
<td>294.28</td>
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<tr>
<td>Benzoic acid</td>
<td>50.75</td>
<td>74</td>
<td>19.70</td>
<td>144.76</td>
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<td>3-(4-OH)propionic acid</td>
<td>44.32</td>
<td>159</td>
<td>0.59</td>
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<td>3,4-DiOH-phenylacetic acid</td>
<td>25.82</td>
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<td>0.47</td>
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<td>4-OH-phenylacetic acid</td>
<td>21.84</td>
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<tr>
<td>4,OH,3-MeOH-cinnamic acid (ferulic acid)</td>
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<td>0.64</td>
<td>108.11</td>
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<td>3,4-DiOH-propionic acid</td>
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<td>0.71</td>
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<td>2,5-DiOH-benzoic acid</td>
<td>5.36</td>
<td>96</td>
<td>0.01</td>
<td>16.97</td>
</tr>
<tr>
<td>Catechol</td>
<td>4.97</td>
<td>94</td>
<td>0.31</td>
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<td>3-OH,4-MeOH-cinnamic acid (isoferulic acid)</td>
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<td>16.40</td>
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<td>114</td>
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<td>4-OH,3-MeOH-benzoic acid (vanillic acid)</td>
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<td>188</td>
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<tr>
<td>3-O-Methylgallic acid</td>
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<tr>
<td>1,3,5-TriMeOH-benzene</td>
<td>2.24</td>
<td>99</td>
<td>0.29</td>
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<td>1,2,3- or 1,2,4-TriOH-benzene</td>
<td>1.70</td>
<td>98</td>
<td>0.00</td>
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<td>3,4-DiOH-benzoic acid</td>
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<tr>
<td>3-OH,4-MeOH-benzoic acid (isovanillic acid)</td>
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<td>144</td>
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<td>3,4-DiMeOH-benzoic acid</td>
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</tr>
</tbody>
</table>

1 Derivatized samples were injected into an Agilent gas chromatography-mass spectrometry system and analyzed with selected-ion monitoring. Concentrations were calculated with a standard calibration curve obtained with pure phenolic standards analyzed under identical conditions. OH, hydroxy; MeOH, methoxy. The following compounds were present at mean concentrations of < 0.1 µmol/L: 3,4-diMeOH-phenol, 3,5-diMeOH-phenol, 1,3,5-triOH-benzoic acid (phloroglucinol), 2-MeOH-benzoic acid, 3-MeOH-benzoic acid, 1,2-diOH,3-MeOH-benzene, 1,4-diOH,3-MeOH-benzene, 2,3-diMeOH-benzoic acid, 3,5-diMeOH-benzoic acid, 2,5-diMeOH-benzoic acid, 2,6-diOH-benzoic acid, 2,3,4-triMeOH-benzoic acid, 2,4,5-triMeOH-benzoic acid, 2-MeOH-phenylacetic acid, 3,4-diMeOH-phenylactic acid, 2-MeOH-phenylpropionic acid, and 2-OH-cinnamic acid (o-coumaric acid).
supplements are taken (147, 148). γ-Tocopherol can, for example, scavenge reactive oxygen and nitrogen species (149, 150) and inhibit cyclooxygenase (151). Additional work is needed to examine the role of tocopherols and tocotrienols in the GI tract.

CONCLUSIONS

Despite the enormous interest in flavonoids and other polyphenolic compounds as potential protective agents against the development of human disease, the real contributions of such compounds to health maintenance and the mechanisms through which they act are still unclear. The frequently proposed systemic antioxidant effects of flavonoids are not supported by strong consistent evidence in vivo. In our view, greater attention should be given to the biological effects of these compounds and their metabolites within the GI tract and to any possible effects on other tissues of flavonoid metabolites (eg, methylated, sulfated, and glucuronidated compounds) generated systemically, as well as products of colonic microbial metabolism that are absorbed.

We thank Prof Ong Choon Nam for help with the provision of data in Table 3 and Pernilla Karlsson and Theo de Kok for contributing some of the fecal water samples.

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Polyphenols and glutathione synthesis regulation

Jan Ø Moskaug, Harald Carlsen, Mari CW Myhrstad, and Rune Blomhoff

ABSTRACT
Polyphenols in food plants are a versatile group of phytochemicals with many potentially beneficial activities in terms of disease prevention. In vitro cell culture experiments have shown that polyphenols possess antioxidant properties, and it is thought that these activities account for disease-preventing effects of diets high in polyphenols. However, polyphenols may be regarded as xenobiotics by animal cells and are to some extent treated as such, i.e., they interact with phase I and phase II enzyme systems. We recently showed that dietary plant polyphenols, namely, the flavonoids, modulate expression of an important enzyme in both cellular antioxidant defenses and detoxification of xenobiotics, i.e., \( \gamma \)-glutamylcysteine synthetase.

This enzyme is rate limiting in the synthesis of the most important endogenous antioxidant in cells, glutathione. We showed in vitro that flavonoids increase expression of \( \gamma \)-glutamylcysteine synthetase and, by using a unique transgenic reporter mouse strain, we showed increased expression in vivo, with a concomitant increase in the intracellular glutathione concentrations in muscles. Because glutathione is important in redox regulation of transcription factors and enzymes for signal transduction, our results suggest that polyphenol-mediated regulation of glutathione alters cellular processes. Evidently, glutathione is important in many diseases, and regulation of intracellular glutathione concentrations may be one mechanism by which diet influences disease development. The aim of this review is to discuss some of the mechanisms involved in the glutathione-mediated, endogenous, cellular antioxidant defense system, how its possible modulation by dietary polyphenols such as flavonoids may influence disease development, and how it can be studied with in vivo imaging.


KEY WORDS Polyphenols, blueberries, glutathione, \( \gamma \)-glutamylcysteine synthetase, gene regulation

OXIDATIVE STRESS AND ANTIOXIDANTS

The hallmark of oxidative stress is increased production of reactive oxygen species (ROS), in amounts that exceed cellular antioxidant defenses. The consequence of oxidative stress may be oxidative damage of lipids, proteins, and DNA, with subsequent disease development and aging (1). ROS production may result from exogenous factors such as radiation and drug exposure or endogenous factors such as increased mitochondrial respiration and oxidative enzymes in infections and inflammation. A possible mechanism for the protective effects of fruits and vegetables with respect to disease is that bioactive compounds in these food items reduce oxidative stress. Fruits and vegetables contain several thousand structurally diverse phytochemicals, of which a large fraction are polyphenols (2). Many polyphenols have antioxidant properties (i.e., reductants) and may react directly with reactive chemical species, forming products with much lower reactivity. Alternatively, compounds in a plant-based diet may increase the capacity of endogenous antioxidant defenses and modulate the cellular redox state. Changes in the cellular redox state, conveying physiologic stimuli through regulation of signaling pathways, may have wide-ranging consequences for cellular growth and differentiation (3). In addition, it has been well documented that polyphenols modulate protein kinase activities (4), serve as ligands for transcription factors (5), and modulate protease activities (6).

Many dietary polyphenols are antioxidants, and the possibility exists that they protect against oxidative damage by directly neutralizing reactive oxidants. We recently measured antioxidant capacity (i.e., electron- or hydrogen-donating capacity) systematically in a variety of dietary plants, including various fruits, berries, vegetables, cereals, nuts, spices, and pulses used throughout the world. Our results (7) demonstrated a > 1000-fold difference in total antioxidant capacity among dietary plants. Interestingly, berries (particularly blackberries, blueberries, and elderberries) high in antioxidant capacity are among the plants with the highest concentrations of polyphenols. This is intriguing, because many berries have been shown to protect against oxidative stress-related pathologic conditions in vivo. For example, Joseph et al (8, 9) demonstrated that long-term feeding of blueberries to rats retarded and even reversed the onset of age-related neurologic dysfunctions, such as a decline in neuronal signal transduction, and cognitive, behavioral, and motor deficits. Stoner and coworkers (10, 11) showed that supplementation with black raspberries in the diet reduced the multiplicity and incidence of esophageal tumors in \( N \)-nitrosomethylbenzylamine-treated rats.

POLYPHENOLS AND \( \gamma \)GCSh GENE REGULATION

The most important endogenous antioxidant defense systems are composed of the thiol-containing tripeptide glutathione and...
small thiol-containing proteins such as thioredoxin, glutaredoxin, and peroxiredoxin. Of these, glutathione is found at millimolar concentrations in most cells and is the major contributor to the redox state of the cell. Glutathione exists in cells in both a reduced form (GSH) and an oxidized form (GSSG); it may also be covalently bound to proteins through a process called glutathionylation (12, 13). The ratio of GSH to GSSG is determined by the overall redox state of the cell. Glutathione is synthesized enzymatically by γ-glutamylcysteine synthetase (γGCS) and glutathione synthetase, with the former being the rate-limiting enzyme (14).

One important task for cellular glutathione is to scavenge free radicals and peroxides produced during normal cellular respiration, which would otherwise oxidize proteins, lipids, and nucleic acids. One mechanism operating to counteract oxidative damage involves transactivation of genes encoding enzymes that participate in glutathione metabolism and synthesis. Typically, these enzymes belong to the phase I and II families of detoxification genes. Analyses of promoter regions of these enzymes suggest that several gene response elements may be involved in such transcriptional regulation, including xenobiotic response elements and antioxidant/electrophil response elements (AREs/EpREs) (15, 16). The latter is defined by a specific consensus sequence of nucleotides (17) and responds to substances with antioxidant properties (18).

Polyphenols are among the most abundant phytochemicals in human food items and, of these, flavonoids are probably the most well studied. The mechanism of action of flavonoids in cellular processes is still not clearly understood. In a series of experiments, we showed that relatively low concentrations of flavonoids stimulated transcription of a critical gene for GSH synthesis in cells (19). Transcriptional control of the catalytic subunit of γGCS, γGCS, is mediated by a 5′ flanking region containing several response elements, including AP-1 sites, one NF-κB site, and several AREs/EpREs (20). Both onion extracts and pure flavonoids transactivated γGCS through antioxidant response elements in the promoter in both COS-1 cells (19) and HepG2 cells (Figure 1), with quercetin being the most potent flavonoid. Structurally similar flavonoids were not as potent; myricetin, with only one hydroxyl group more than quercetin, was inactive, which emphasizes the apparent specificity of γGCS induction.

Flavonoids in food items are conjugated to various sugar molecules, which likely influence their intestinal absorption, transport, and entry into cells. Conjugates of quercetin were found to be totally inactive in tissue cell cultures (19), which emphasizes the importance of in vivo experiments, in which the activity of tested substances reflects bioavailability, metabolism, cellular activity, and excretion of micronutrients. To this end, we developed a transgenic mouse strain with ∼3.8 kilobases of the γGCS promoter upstream of luciferase incorporated into its genome. We used this mouse strain to test the ability of polyphenol-rich berries to modulate γGCS gene expression, with a novel in vivo whole-body or ex vivo whole-organ imaging technique (20) and analysis of gene expression in tissue homogenates and sections (21) (Figure 2). For monitoring of basal γGCS promoter activity in the transgenic mice, β-luciferin was injected intravenously and the mice were placed on their dorsal sides in a light-sealed chamber and underwent imaging with an ultra-sensitive video camera. Strong luminescence was detected from the whole animal, reflecting ubiquitous expression of γGCS and glutathione synthesis. Several organs were then excised and subjected to ex vivo imaging (Figure 2). Interestingly, strong luminescence was emitted from brain, muscle, and epididymis/testis, whereas kidney and lung demonstrated moderate luminescence and liver, spleen, and heart showed much lower activity. When transgenic animals were fed juice or homogenates made from antioxidant-rich berries for 3–4 wk, we observed that γGCS promoter activity was modulated in organs such as skeletal muscle, brain, and liver (21). We also measured glutathione concentrations in the organs in which modulation of the γGCS promoter was observed. The only organ in which we could detect statistically significant increases in total glutathione concentrations was gastrocnemius muscle (Figure 3).

In vivo feeding experiments with polyphenol-rich diets revealed large differences in γGCS promoter activity responses among individual animals. Some animals responded and some did not. One possible explanation for this phenomenon may be related to differences in bacterial populations in the gut microbial flora influencing the extent of enzymatic hydrolysis of polyphenol conjugates. Indeed, Aura et al (22) showed that conjugates of flavonoids are hydrolyzed by the gut flora among humans, and several studies demonstrated that sugar moieties influence enterocyte uptake and thus tissue distribution (reviewed in ref. 23).
of polyphenols are mediated through one or several Nrf2 binding sites (ie, AREs/EpREs), several activities of flavonoids could be responsible for the effect on the γGCSH promoter. Nrf2 is activated through cytosol sequestering through binding to the cytoskeleton-associated protein Keap1. Binding of Nrf2 to Keap1 is thought to be dependent on critical thiols in Keap1, thiols that are possibly sensitive to the cellular redox state. Therefore, release and subsequent translocation of Nrf2 to the nucleus are presumably sensitive to cellular oxidative stress, thiol-reactive compounds, and antioxidants (24) (Figure 4). Because ARE/EpRE-containing gene promoters are thought to be regulated by substances with antioxidant properties, a possible explanation for the different γGCSH promoter–inducing activities could be that some flavonoids are better antioxidants than others. However, when we measured the ability of flavonoids to reduce Fe³⁺ in the ferric ion-reducing activity assay, which is commonly used to measure total antioxidant capacity in biologic samples (25), we found that the antioxidant capacity did not correlate with γGCSH-inducing activity in COS-1 cells. Myricetin had a higher antioxidant capacity than did kaempferol but it did not transactivate the γGCSH promoter (19) (Figure 5), which suggests that transcriptional γGCSH regulation depends on other properties of the flavonoids.

Several activities of flavonoids should be considered in this respect. First, flavonoid antioxidant scavenging of free radicals often involves formation of a radical of the flavonoid itself. Quercetin is oxidized to a quinone when serving as an antioxidant, and Boot et al (26) recently showed that such quinones react with thiols. Therefore, it could be speculated that free radical-oxidized quercetin reacts with thiols in Keap1, the key regulatory protein in transcriptional regulation of antioxidant-responsive genes through Nrf2.

Second, the chemical properties of some antioxidants may also give them prooxidant properties (27), and this should be considered with respect to mechanisms for induction of cellular antioxidant defenses. Flavonoids can auto-oxidize (27), and products of auto-oxidation can possibly react with or otherwise reduce cellular concentrations of glutathione. Quercetin and myricetin are known to auto-oxidize at physiologic pH (28), and subsequent reduction of glutathione concentrations can possibly explain transcriptional up-regulation of both γGCS subunits (29).

Third, it can be speculated that the effect is mediated by increased mitochondrial production of hydrogen peroxide and superoxide anion. Hodnick et al (30) showed that quercetin and myricetin cause mitochondrial respiratory bursts. However, myricetin was found to be the most efficient enzyme inhibitor and producer of mitochondrial respiratory bursts, whereas kaempferol was the least efficient and quercetin was intermediate. Therefore, there seems to be no correlation between the ability to
modulate mitochondrial respiration and the γGCSH promoter induction observed in our studies (19).

The aforementioned explanations depend on potentially deleterious effects of flavonoids. How deleterious effects may be translated into disease-preventing effects may not be obvious, but the principle of hormesis has been suggested to explain how repeated challenges, such as repeated low oxidative stress, may adapt cells and organs and in the long term make them more resistant to severe oxidative stress (31). The hormetic principle has been proposed to explain why physical activity and exercise, which increase oxidative stress in various tissues, can significantly decrease the risk of disease development (32). It is possible that repeated mild cellular oxidative stress induced by flavonoids through the diet boosts cellular antioxidant defense systems and in the long term shifts these defense systems to a higher steady state, which prevents disease development or reduces the impact of oxidative stress when disease occurs. Nrf2-mediated transactivation is also influenced by phosphorylation (33), making it possible that the protein kinase inhibitory activities of flavonoids may be involved.

**CELLULAR EFFECTS OF ALTERED GLUTATHIONE METABOLISM**

Detoxification of xenobiotics requires sufficient glutathione synthesis for conjugation and excretion of GSH-conjugated metabolites. Therefore, polyphenol-stimulated glutathione synthesis could well be beneficial in cellular handling of carcinogenic substances, for example. The role of glutathione in detoxification was recently reviewed (34).

The intracellular glutathione concentration and the redox status of the cell are likely to influence regulation of protein function through glutathionylation, ie, covalent binding of glutathione to the protein through disulfide bonds. Until recently, glutathionylation of proteins had been described for only a few cellular proteins. A proteomic approach taken by Fratelli et al (35) identified 38 proteins that are glutathionylated in oxidatively stressed T lymphocytes.

Two mammalian transcription factors known to be glutathionylated are AP-1 and NF-κB (36), but the role of such covalent modification in the cell nucleus is still under debate. Determination of the roles of transcription factors in the regulation of many disease-associated genes in cancer and chronic inflammatory diseases requires detailed studies to establish the influence of cellular glutathione on their activity. Many transcription factors contain thiol groups that have been shown to influence DNA binding. Redox switching and regulation of transcription factors were recently described in great detail, particularly for single-cell organisms (37). Because glutathione is the fundamental redox regulator in eukaryotic cells, it is conceivable that principles of GSH-mediated redox switching of transcription factors can be extrapolated from single cells to multicellular organisms.
Neurodegenerative diseases such as Parkinson’s disease, involving dopaminergic neurons, are associated with neuronal oxidative stress. It was recently shown that the dopamine-synthesizing enzyme tyrosine hydroxylase is inhibited by reversible glutathionylation during oxidative stress (38).

Cellular turnover of proteins is mediated to a large extent by proteasomes. Recent evidence from Demasi and Davis (39) showed that protein turnover attributable to proteasomes is regulated by intracellular glutathione. Those authors found that glutathionylation of 20 S proteasomes and hydrogen peroxide-induced oxidative stress inhibited the proteolytic activity of proteasomes.

Enzymes such as protein kinases and phosphatases have been shown to be modulated by critical thiol groups (for a recent review, see ref 40), and protein kinase A can be regulated directly through glutathionylation (41). Intracellular concentrations of glutathione clearly play a role in the regulation of protein tyrosine phosphatase SHP-1, which is oxidized and inactivated by hydrogen peroxide and can be re-reduced and activated by thiols such as glutathione (42).

Intracellular glutathione also influences immune responses. Production of major immune regulatory cytokines such as interleukin-12 has been shown to be regulated by intracellular glutathione (43). Even cell cycle transitions involving cyclin D1 appear to be redox regulated, because thiol-containing antioxidants delay the transition from G0 to G1 and S phases in embryonic mouse cells (44).

**GLUTATHIONE AND DISEASE**

Oxidative stress is a general term intimately linked to production of reactive oxygen, nitrogen, or iron species and the overall redox state of the cell. Oxidative stress can be assessed by measuring GSH and GSSG, and it is frequently expressed as the ratio between the two. Indeed, the ratio has been suggested as a clinical marker in diseases in which oxidative stress is particularly important. Interestingly, GSH is oxidized to GSSG in an age-dependent manner, possibly reflecting accumulating oxidative stress (45). A decreased ratio between GSH and GSSG is also associated with progression of tumors (46), and decreased total glutathione concentrations were found among patients with chronic diseases, including gastrointestinal, cardiovascular, and musculoskeletal diseases (47). Oxidative stress in general and GSH concentrations in particular are also associated with neurodegenerative disorders and HIV. In Parkinson’s disease, ROS concentrations are increased in parts of the substantia nigra and glutathione concentrations are decreased (48). Some improvement among patients with early Parkinson’s disease has been observed with administration of glutathione (49). Decreased concentrations of glutathione among HIV-infected individuals may have dual effects. First, decreased concentrations are known to promote apoptosis in the CD4+ subtype of T cells (50). Second, it has been shown that intracellular thiols regulate HIV transcription (51) and that GSH increases transcriptional activation of HIV through NF-κB binding sites in the HIV long terminal repeat (52). These combined effects suggest that decreased concentrations contribute significantly to HIV/AIDS development and that supplementation with glutathione precursors (such as N-acetylcysteine) may be of some benefit for HIV/AIDS patients. Corroborating this, Herzenberg et al (53) found that GSH concentrations were associated with survival rates in HIV. Other viruses also seem to be dependent on the intracellular redox state for replication. Expression of late influenza viral proteins was found to be increased when cells were treated to reduce intracellular glutathione concentrations (54).

Alteration of glutathione concentrations may also be associated with tumor development through dependence on glutathione S-transferases and glutathione peroxidases. These enzymes are essential in detoxification of carcinogens and scavenging of ROS. Polymorphisms in various classes of glutathione S-transferases have been associated with increased development of tumors of the lung, bladder, and colon (55) and head and neck (56). Decreased activity of key enzymes involved in glutathione synthesis, accompanied by decreased availability of cyst(e)ine synthesis, contributes to mucosal glutathione deficiency in inflammatory bowel disease (57). Glutathione is also critical in the lung, and altered concentrations have been associated with pulmonary diseases such as acute respiratory distress syndrome (58) and chronic obstructive pulmonary disease (59).

**CONCLUDING REMARKS**

Dietary antioxidants have long been suspected to scavenge reactive ROS and thereby avert deleterious effects on proteins, lipids, and nucleic acids in cells. This has been put forward as one of the major mechanisms for the disease-preventing effects of fruits and vegetables. Our results (19, 21) add modulation of intracellular GSH concentrations to the list of possible disease-preventing effects of polyphenols, with the implication that they modulate GSH-dependent cellular processes, such as detoxification of xenobiotics, glutathionylation of proteins, and regulation of redox switching of protein functions in major cellular processes.

The potential beneficial effects of flavonoids have prompted commercial interest in manufacturing supplements containing high concentrations of flavonoids. However, their concentration-dependent cellular effects, some of which may be harmful, should be of concern with respect to high-dose supplementation. Flavonoids at high concentrations produce reactive radicals through auto-oxidation and increasing mitochondrial respiratory bursts (27, 30). Although the redox potentials of most flavonoid radicals are lower than those of the superoxide and peroxyl radicals (60), the effectiveness of the radicals in generating lipid peroxidation, DNA adducts, and mutations may still be significant in disease development (61). Also of concern is the observation that some flavonoids inhibit enzymes (such as topoisomerases) involved in DNA structure and replication (62), and it has been suggested that high intake of flavonoids predisposes subjects to the development of certain childhood leukemias (63, 64). Flavonoid supplementation as a general recommendation to increase cellular glutathione concentrations may also be troublesome, because glutathione has a major role in overall redox regulation of cell functions and is not suitable as a therapeutic target for substances that alter cellular concentrations by orders of magnitudes. Therefore, the current recommendation is to increase fruit and vegetable consumption. Because the active compounds and the mechanisms involved in disease-preventing effects are still poorly understood, the recommendation is that eating a variety of fruits and vegetables provides the best protection. It remains to be determined whether dietary polyphenols modulate cellular glutathione concentrations among humans and whether they contribute to regulation of major cellular signaling.
pathways, which would explain the indisputable fact that fruits and vegetables protect against disease.

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Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations

Joshua D Lambert, Jungil Hong, Guang-yu Yang, Jie Liao, and Chung S Yang

ABSTRACT
Many plant polyphenolic compounds have been shown to have cancer-preventing activities in laboratory studies. For example, tea and tea preparations have been shown to inhibit tumorigenesis in a variety of animal models of carcinogenesis, involving organ sites such as the skin, lungs, oral cavity, esophagus, stomach, liver, pancreas, small intestine, colon, and prostate. In some of these models, inhibitory activity was demonstrated when tea was administered during the initiation, promotion, or progression stage of carcinogenesis. The cancer-preventing activities of these and other polyphenols, such as curcumin, genistein, and quercetin, are reviewed. In studies in vitro, many of these compounds have been shown to affect signal transduction pathways, leading to inhibition of cell growth and transformation, enhanced apoptosis, reduced invasive behavior, and slowed angiogenesis. However, the concentrations used in cell culture studies were much higher than those found in vivo. If we propose mechanisms for cancer prevention on the basis of cell line experiments, then these activities must be demonstrated in vivo. The bioavailability, ie, tissue and cellular concentrations, of dietary polyphenols is a determining factor in their cancer-preventing activity in vivo. For example, compounds such as curcumin are effective when applied topically to the skin or administered orally to affect the colon but are not effective in internal organs such as the lungs. More in-depth studies on bioavailability should facilitate correlation of mechanisms determined in vitro with in vivo situations, increase our understanding of dose-response relationships, and facilitate extrapolation of results from animal studies to human situations. Am J Clin Nutr 2005;81(suppl):284S–91S.

KEY WORDS Tea polyphenols, green tea, curcumin, genistein, quercetin, tumorigenesis

INTRODUCTION
Polyphenolic compounds constitute one of the largest and most ubiquitous group of phytochemicals. They are formed to protect plants from photosynthetic stress, reactive oxygen species, and herbivory. Polyphenolic compounds are an important part of the human diet, with flavonoids and phenolic acids being the most commonly occurring ones in food. Early interest in polyphenols was related to their “antinutritional” effects, ie, decreasing absorption and digestibility of food through their ability to bind proteins and minerals. The astringency of many fruits and beverages is attributable to the precipitation of salivary proteins by plant polyphenols. Current interest is focusing on the beneficial health effects of dietary polyphenols, because these compounds may have antioxidative, antiinflammatory, and anticarcinogenic activities. The chemical properties, biochemical properties, and bioavailabilities of many polyphenolic compounds have been reviewed (1). This article updates these subjects, with emphasis on the potential cancer-preventing activities of dietary polyphenols and the possible mechanisms involved. We review the data on the tea polyphenols, curcumin, genistein, and quercetin (Figure 1). A more extensive discussion of the tea polyphenols is used to illustrate the key issues related to cancer prevention.

TEA AND ITS CANCER-PREVENTING ACTIVITIES

Tea constituents

Tea (Camellia sinensis) is a warm-weather evergreen. In the making of green tea, the tea leaves are heated to inactivate the enzymes. Therefore, the constituents are preserved in the dried tea leaves. A typical brewed green tea beverage (eg, 2.5 g tea leaves in 250 mL hot water) contains 240–320 mg of catechins and 20–50 mg of caffeine. The catechins include (–)-epicatechin, (–)-epigallocatechin, (–)-epicatechin-3-gallate, and (–)-epigallocatechin-3-gallate (EGCG), with EGCG being the major catechin in tea (Figure 1). Tea leaves also contain flavonols such as quercetin and myricetin, as well as the nitrogenous compounds caffeine (2–5% by wt) and theobromine (2). During the production of black tea, the tea leaves are withered, crushed, and allowed to undergo an enzyme-mediated oxidation commonly referred to as fermentation. During this process, much of the catechin content of the tea leaves is converted to dimeric theaflavin molecules and polymeric thearubigins. In brewed black tea, catechins, theaflavins, and thearubigins account for 3–10%, 2–6%, and > 20% of the dry weight, respectively (Figure 1). Theaflavins are responsible for the color of and contribute to the taste of black tea.

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3 Supported by NIH grant CA88961.
4 Address reprint requests and correspondence to CS Yang, Susan Lehman Cullman Laboratory for Cancer Research, Ernest Mario School of Pharmacy, Rutgers, State University of New Jersey, 164 Frelinghuysen Road, Piscataway, NJ 08854. E-mail: csyang@rci.rutgers.edu.
Inhibition of carcinogenesis in animal models by tea and its constituents

Cancer-preventing activity has been demonstrated for green tea, black tea, and their constituents in many animal models of tumorigenesis, including the skin, lung, oral cavity, esophagus, stomach, liver, pancreas, bladder, small intestine, colon, and prostate gland (3–5). Table 1 summarizes the results of some of those studies.

In most studies, different preparations of tea or tea constituents were administered in drinking water; these included freshly brewed green or black tea (e.g., 5-20 g of leaves brewed in 1 L of water, referred to as 0.5-2% tea); green or black tea solids (dehydrated tea extracts), usually reconstituted with distilled water at concentrations of 0.4% or 0.6%; decaffeinated green or black tea solids prepared as described above; or green tea or black tea polyphenol preparations (which are enriched in tea polyphenols but also may contain some caffeine). The tea preparations were administered either during or after the carcinogen treatment period or during the entire experimental period. Tea preparations were found to be effective in inhibiting 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorigenesis in A/J mice with all 3 of the dosing schedules listed above (5). Inhibition of tumor multiplicity (number of tumors per mouse) was observed in most of the studies, although inhibition of tumor incidence (number of animals with tumors) was reported in some publications. In the NNK-induced lung tumorigenesis model, administration of a black tea preparation to adenoma-bearing mice significantly inhibited tumor cell proliferation and the progression of adenoma to carcinoma (24). Inhibition of tumor invasion and metastasis in transplanted and spontaneous metastasis models by orally administered green tea infusion or EGCG was also reported (25, 26).

Other experiments did not demonstrate inhibition of lung tumorigenesis by tea preparations. For example, Witschi et al (27) reported that green tea extract did not reduce lung tumor multiplicity in male A/J mice treated with a single dose of NNK or in a cigarette smoke-induced lung tumorigenesis model.

Whereas most of the studies were conducted with chemical- or ultraviolet (UV) light–induced tumorigenesis models, tea has also been shown to inhibit spontaneous tumorigenesis. For example, administration of 1% or 2% freshly brewed green or black tea significantly inhibited the spontaneous development of lung adenoma and rhabdomyosarcoma in A/J mice (28). In that

<table>
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experiment, as well as in some other experiments, the tea-treated groups had lower body weight and body fat than did the control group, although food consumption was not significantly different between the groups. For example, with 1% green tea in the aforementioned experiment, the body weights and retroperitoneal fat pad weights were 14% and 35% lower, respectively, than those for the control group (28). It remains unclear whether these differences in body weight and fat are related to the mechanisms of cancer prevention or are simply coincidental.

With the TRAMP mouse model of prostate carcinogenesis, it was recently demonstrated that oral consumption of 0.1% green tea polyphenols decreased tumor incidence by 65%. In contrast to water-treated animals, which demonstrated a high rate of distal metastasis (25–95%), tea-treated mice showed no distal metastasis. Biochemical and histologic analyses showed a significant decrease in proliferating cell nuclear antigen and a 10-fold increase in tumor cell apoptosis (6). Inhibition of matrix metalloproteinase (MMP)-2 and MMP-9, as well as vascular endothelial growth factor (VEGF), was also observed (29).

The bioavailability of tea constituents is apparently a key factor determining the effectiveness of tea in inhibiting tumor formation. In this respect, the oral cavity and digestive tract, which have direct contact with orally administered tea, may represent good targets for chemoprevention. In the 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis hamster model, treatment with 0.6% green tea, as the sole source of drinking fluid, reduced the number of visible tumors by 35% and reduced tumor volume by 57%. In addition, immunohistochemical analyses showed that tea increased the apoptotic index of the tumors while decreasing the proliferation index and microvessel density (20). Tea preparations were also shown to inhibit esophageal, forestomach, and intestinal cancer (5). Inhibition of colon cancer was observed by some investigators but not others (5).

Purified tea constituents have also been reported to inhibit tumorigenesis. For example, EGCG inhibited lung tumorigenesis in A/J mice induced by NNK and cisplatin (30, 31). Theaflavins (a mixture of theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate [TFdiG]) reduced NNK-induced lung tumor multiplicity and volume in A/J mice (32). These findings are interesting, given the extremely poor bioavailability of theaflavins, and may suggest that the theaflavins are metabolized to a more-bioavailable active metabolite. In NNK-treated F344 rats, the inhibitory activity of black tea could be fully accounted for by the caffeine content of the tea (33). Caffeine has also been shown to play an important role in the inhibition of skin carcinogenesis. For example, Huang et al (34) reported that, whereas orally administered green tea and black tea were effective in reducing the incidence and multiplicity of UVB light-induced skin tumors, orally administered decaffeinated teas were much less effective. The addition of caffeine restored the activity of the decaffeinated teas. A recent study showed that topical application of caffeine or EGCG to SKH-1 hairless mice that had been pretreated twice weekly for 20 wk with UVB light decreased the multiplicity of skin tumors by 44–72% or 55–66%, respectively. In addition, both compounds were shown to increase the apoptotic index of the tumors by 56–92%, as measured with immunohistochemical assays for caspase-3–positive cells (21). The inhibition of skin tumorigenesis by caffeine or tea was shown to be closely correlated with the reduction of body fat (24).

**Mechanistic studies in vitro**

**Antioxidant/prooxidant activity**

Numerous potential mechanisms have been proposed for the cancer-preventing activity of tea and tea constituents, on the basis of studies with cancer cell lines. One problem encountered in most studies is that the concentrations of tea compounds used usually exceed those found in animal or human plasma or tissues after tea consumption.

Tea polyphenols have been shown to have strong antioxidant activity in vitro, but such activity was associated with inhibition of tumorigenesis in only a few cases (30, 35). The importance of this potential mechanism in vivo remains unclear. In contrast, some studies suggested that the cell-killing activity of these compounds, at least in vitro, might be related to their prooxidant activity. For example, we showed that EGCG-induced apoptosis, but not growth inhibition, in H661 lung cancer cells and Ras-transformed human bronchial cells was completely or partially blocked with the inclusion of catalase in the medium (36, 37).

When EGCG was added to cell culture systems (e.g., HT29 cells in McCoy’s 5A medium), production of H₂O₂ was observed (38). Preincubation of cells with EGCG (before the addition of a growth factor) was shown to block the epidermal growth factor (EGF)- and platelet-derived growth factor–induced signaling systems (39). Our recent results with KYSE510 esophageal squamous cells indicated that inhibition of the EGF signaling system was associated with degradation or inactivation of the EGF receptor, and the effect could be abolished with the inclusion of superoxide dismutase (Z Hou and CS Yang, unpublished results, 2004). The addition of superoxide dismutase also stabilized EGCG, suggesting that the effect was not attributable to competition between EGCG and EGF for the EGF receptor or inhibition of the activated receptor tyrosine kinase activity by EGCG. These observations suggest a role for EGCG prooxidation in some of the observed activities of EGCG in vitro. It is unclear whether such reactions occur in vivo.

**Specific protein kinases**

Enhanced activity of the transcription factors activator protein 1 (AP-1) and nuclear factor κB (NF-κB) is key in carcinogenesis. EGCG and other tea polyphenols have been shown to inhibit the activation of AP-1 and NF-κB. Although antioxidative mechanisms have been implicated in this activity, the results can be better explained by the direct inhibition of specific protein kinases by these tea polyphenols.

Treatment of 30.7b Ras 12, Ras-transformed, mouse epidermal cells with 20 μmol/L EGCG and theaflavins was shown to decrease amounts of phosphorylated Erk 1/2 and Mek 1/2 (40), as well as the association between Raf-1 (an upstream protein kinase) and Mek1. TFdiG and EGCG were also shown to directly inhibit the kinase activity of Erk1/2 by competing with Elk-1 for access to the active site (40). Similar results were observed after treatment of Ras-transformed human bronchial cells with EGCG and TFdiG (37).

EGCG and TFdiG were shown to inhibit the activity inhibitor κB (IκB) kinase in tumor necrosis factor-α-stimulated IEC-6 intestinal epithelial cells and lipopolysaccharide-stimulated RAW 264.7 murine macrophages (41, 42). EGCG and theaflavins were also reported to inhibit cdk2 and cdk4, leading to the hyperphosphorylation of retinoblastoma protein, which is expected to result in G1/G0 phase arrest (43).
Enhancement of apoptosis

EGCG was reported to induce apoptosis of cultured cells (5). As discussed previously, the H$_2$O$_2$ generated in the cell culture system through autoxidation of EGCG could account for a large proportion of the reported activity. A recent study suggested that certain green and black tea polyphenols bind to the antiapoptotic proteins Bcl-2 and Bcl-xL and thus may induce apoptosis (44). The study used a combination of nuclear magnetic resonance binding assays, fluorescence polarization assays, and computational docking studies. The results, with very low (nanomolar) concentrations of EGCG, were very impressive but must be confirmed in whole cells. To date, apoptosis has been observed only with much higher doses of EGCG (20–100 µmol/L) (5).

Inhibition of angiogenesis

Cao and Cao (45) demonstrated inhibition of endothelial growth and angiogenesis in the chorioallantoic membrane assay with EGCG (20 µmol/L). Those authors also showed that oral administration of 1.25% green tea to mice inhibited corneal neovascularization stimulated by VEGF (45). EGCG was also shown to inhibit the expression of VEGF by head and neck squamous cell, breast, and colon carcinoma cells (13, 46, 47).

Inhibition of other enzymes

EGCG was shown to selectively inhibit the activity of topoisomerase I, but not topoisomerase II, in human colon cancer cell lines at concentrations that effectively inhibit cell growth (48). Inhibition of MMP-2 and MMP-9 by EGCG was also demonstrated at relatively low doses (49). Others showed that EGCG similarly inhibited the activity and expression of membrane type 1 MMP, a protein responsible for the activation of MMPs (50). Because of the physiologically achievable concentrations used, these activities represent an attractive mechanism for the observed antiangiogenic activity of EGCG and green tea in vivo (5).

EGCG has been reported to inhibit the chymotryptic activity of 20S proteasome in leukemic, breast cancer, and prostate cancer cell lines (51). This inhibition causes accumulation of p27Kip1 and IκB, which results in G$_0$/G$_1$ phase cell cycle arrest and inhibition of NF-κB activity, respectively. The 50% inhibitory concentration for EGCG-mediated inhibition of proteasomes in cells (1–10 µmol/L) is 10-fold higher than the 50% inhibitory concentration in a cell-free system (86-194 nmol/L). This is likely attributable to the nonspecific binding of EGCG to other cellular components. Such nonspecific binding may lead to a higher dose requirement in vivo.

CURCUMIN

Topical application of 10 mmol curcumin to the cheek pouch of Syrian golden hamsters was shown to reduce the number of visible DMBA-induced oral tumors by 39.6% and the tumor volume by 61.3% (20). Administration of 4% curcumin in the diet of azoxymethane-treated mice during the initiation period reduced the number of colon tumors by 66%. If treatment was started after initiation, then tumor number was reduced by 25% (52). In APC$^{min}$ mice given 0.2% or 0.5% curcumin in the diet, intestinal adenoma multiplicity was reduced by 39–40% and hematocrit amounts, which were reduced in untreated mice, were partially restored (53). Topical application of curcumin has also been shown to reduce tumor incidence and multiplicity in both the benzo[a]pyrene/12-O-tetradecanoyl phorbol-13-acetate (TPA) and DMBA/TPA models of skin carcinogenesis in mice (34, 54).

As with the tea polyphenols, the poor bioavailability of curcumin limits its cancer-preventing activity. Curcumin was found to be ineffective in preventing NNK-induced lung tumorigenesis in A/J mice and liver and kidney tumorigenesis in Long-Evans Cinnamon rats (55, 56). All of these tissues require systemic bioavailability for cancer prevention.

Studies with cell lines have shown that curcumin possesses both antioxidant and prooxidant activities. Curcumin-induced, Akt-mediated apoptosis of Caki renal cells was partially inhibited by cotreatment with N-acetylcyesteine, which suggests the involvement of oxidative stress (57). Cyclooxygenase-2 and inducible nitric oxide synthase have also been reported as potential targets for curcumin (58, 59). Treatment of lipopolysaccharide-treated mice with curcumin (92 µg/kg, administrated intragastrically) reduced hepatic inducible nitric oxide synthase mRNA expression by 50–70% (60).

Curcumin has also been shown to inhibit activation of the transcription factors AP-1 and NF-κB in human leukemia cell lines (61, 62). Ablation by curcumin of AP-1 and NF-κB activation has been demonstrated in TPA-treated mouse skin. Chun et al (63) reported that topical curcumin application to mouse skin inhibited TPA-mediated activation of Erk and p38 mitogen-activated protein kinase and subsequent activation of NF-κB.

It is unclear from the currently available data what the primary target (or targets) of curcumin is. Many but not all of the animal models studied involve inflammatory mediators such as TPA or lipopolysaccharide, which indicates that curcumin may act primarily as an antiinflammatory agent. Additional studies with in vivo biomarkers are needed.

GENISTEIN

Genistein and daidzein (isoflavones derived from soybeans) have been shown to inhibit the development of both hormone-related and non–hormone-related cancers, including mouse models of breast, prostate, and skin cancer. Treatment of TRAMP mice with 100–300 mg genistein/kg diet reduced the incidence of advanced-stage prostate tumors, in a dose-dependent manner (64). A high-isoflavone diet was also shown to inhibit methylnitrosourea-induced prostate tumors in Lobund-Wistar rats (65).

Topically applied genistein was shown to reduce the incidence and multiplicity of skin tumors in the DMBA-initiated and TPA-promoted Sencar mouse model by 20% and 50%, respectively (66). In the UVB light-induced complete carcinogenesis model, topical pretreatment of SKH-1 mice with 10 µmol genistein significantly reduced the formation of H$_2$O$_2$ and 8-hydroxy-2'-deoxyguanosine but not pyrimidine dimers in the epidermis (66).

Treatment of mouse mammary tumor virus-neu mice with diet containing 250 mg/kg genistein or daidzein significantly increased the latency for spontaneous breast tumors but did not affect tumor size or multiplicity (67). In contrast, it was reported that treatment of mice in the DMBA-initiated and medroxyprogesterone acetate-promoted model with diet containing 1 g/kg genistein increased progression of mammary adenomas, compared with control animals (68).

The ability of genistein to inhibit colon cancer in animals remains unclear. In carcinogen-treated rats, both positive and
null effects on the formation of aberrant crypt foci have been reported. No inhibitory effect on intestinal carcinogenesis was observed with APC\textsuperscript{min} mice fed a Western-style (high-fat/low-fiber/low-calcium) diet containing 16–475 mg/kg soy isoflavones (69).

Several mechanisms have been proposed for genistein. Genistein and other isoflavones have demonstrated weak estrogenic activity at lower concentrations but are estrogen receptor antagonists at higher concentrations. At 40 \mu M, genistein inhibited proliferation of LNCaP prostate cancer cells and enhanced apoptosis. Treatment of human bladder cancer cells with genistein resulted in inhibition of cdc2 kinase activity and G2/M phase cell cycle arrest (70). Genistein has been shown to inhibit 20S proteasomal activity in LNCaP and MCF7 cells, resulting in accumulation of p27\textsuperscript{Kip-1}, p16\textsuperscript{INK4a}, and Bax (71). Genistein has also been shown to inhibit tyrosine kinase and topoisomerase activity (72). The relative importance of each of these mechanisms remains to be determined in vivo.

The bioavailability of genistein appears to be better than that of the green tea polyphenols or curcumin (73, 74). Plasma concentrations of genistein in tumor-bearing nude mice fed a diet containing 1 mg/g genistein were 3.4 \mu M (75). Among humans, a single oral dose of 460 mg resulted in peak plasma concentrations of 20–25 \mu M (76). The concentrations achieved in the target tissues must still be accurately determined.

**QUERCETIN**

Studies with animal models of carcinogenesis have yielded mixed results regarding the cancer-preventing activity of quercetin. For example, it was reported that 2% dietary quercetin inhibited azoxymethane-induced hyperproliferation and focal dysplasia in mice (77). Quercetin also reduced tumor incidence by 76% and tumor multiplicity by 48%. However, no inhibitory effects were observed in APC\textsuperscript{min} mice treated with quercetin (78). Similarly, although quercetin inhibited local, UVB light-induced immunosuppression in SKH-1 hairless mice, it had no effect on skin tumorigenesis (79). Quercetin did inhibit N-nitrosodiethylamine-induced lung tumorigenesis in mice when administered during the initiation phase (80).

Mechanistic studies have shown that treatment of colon cancer cells with quercetin results in reduced expression of Ras protein (81). Inhibition of cell growth in pancreatic and colon cancer cells has been correlated with inhibition of EGF receptor expression and tyrosine kinase activity (82, 83). In HL-60 promyelocytic leukemia cells, it was found that the ability of quercetin to damage DNA was largely attributable to the formation of H\textsubscript{2}O\textsubscript{2} (84).

**POSSIBLE MECHANISMS FOR INHIBITION OF CARCINOGENESIS**

Although many biological actions of polyphenols have been described, the mechanisms responsible for inhibition of carcinogenesis in animals remain unclear. One of the problems with extrapolating results of in vitro studies to animals is that the concentrations of test compounds used in vitro usually exceed those found in plasma or tissues after polyphenol consumption; genistein may be an exception. Even when physiologically achievable concentrations are effective, as has been reported for some enzyme systems (44, 49, 51), caution is still needed in the extrapolation from purified enzymes to animals, as we previously discussed. Therefore, any proposed mechanisms must be verified in animal models or human tissues.

Multiple mechanisms appear to be involved in the inhibition of carcinogenesis by dietary polyphenols (Figure 2). The relative importance of these mechanisms depends on the model system used. Tea polyphenols and curcumin have been shown to inhibit key signal transduction protein kinases, such as mitogen-activated protein kinase, I\textsuperscript{κB}, and certain cyclin-dependent kinases. This action would inhibit cell growth and transformation, induce apoptosis, and inhibit angiogenesis. As mentioned above, inhibition of cell growth and induction of apoptosis by tea polyphenols, curcumin, genistein, and quercetin have been noted in
one or more of the following tissues: skin, prostate, colon, and lung. Tea consumption and genistein demonstrated antiangiogenic activity in NNK-induced lung adenoma (J Liao and CS Yang, unpublished observations, 2004) and Lewis lung xenograft (85) mouse models, respectively. Inhibition of arachidonic acid metabolism by tea polyphenols and curcumin has been reported (86–90), and such activity may contribute to the inhibition of carcinogenesis. Other mechanisms, such as inhibition of topoisomerase or inhibition of proteasome, remain to be validated in vivo.

CONCLUSIONS

Because of the extensive consumption of polyphenols in the diet, the biological activity of these compounds is an important topic of scientific investigation. Given the potential cancer-preventing activities of these compounds, we would expect to observe these activities in human populations. However, epidemiologic studies have not provided sufficient evidence for such conclusions. On the basis of what is known about the bioavailability of different dietary polyphenols, it is likely that organ sites that are most accessible to dietary polyphenols experience the protective effects of these compounds. Future studies, especially carefully designed, mechanism-based, animal studies, may facilitate better understanding of the potential health benefits of dietary polyphenols.

REFERENCES


Polyphenols and cardiovascular disease: effects on endothelial and platelet function

Joseph A Vita

ABSTRACT
Epidemiologic studies suggest that higher polyphenol intake from fruits and vegetables is associated with decreased risk for cardiovascular disease. The mechanisms explaining this observation remain unclear. This review summarizes data suggesting that flavonoids improve endothelial function and inhibit platelet aggregation in humans. The vascular endothelium is a critical regulator of vascular homeostasis, and endothelial dysfunction contributes to the pathogenesis and clinical expression of coronary artery disease. Platelet aggregation is a central mechanism in the pathogenesis of acute coronary syndromes, including myocardial infarction and unstable angina. For these reasons, the observed effects of flavonoids on endothelial and platelet function might explain, in part, the observed beneficial effects of flavonoids on cardiovascular disease risk. Am J Clin Nutr 2005;81(suppl):292S–7S.

KEY WORDS Polyphenols, cardiovascular disease, endothelium, platelets, flavonoids, tea

INTRODUCTION
This review represents a summary of a presentation I made at the 1st International Conference on Polyphenols and Health, in Vichy, France, on November 20, 2003. The review provides an overview of epidemiologic data supporting a relationship between higher intake of polyphenolic flavonoids and reduced risk of cardiovascular disease. The remainder of the review focuses on possible mechanisms for this beneficial effect, including improved endothelial function and reduced platelet aggregation.

EPIDEMIOLOGIC STUDIES OF FLAVONOID CONSUMPTION
Many epidemiologic studies have investigated the relationship between flavonoid intake and cardiovascular disease risk, and they have provided mixed results (1–12). The subject has been reviewed (13–17); overall, the evidence suggests that individuals with the highest flavonoid intake have modestly reduced risks for cardiovascular disease (1, 3, 4, 7–9). In some studies, the apparent benefit of flavonoids was quite large. For example, Sesso et al (8) examined the relationship between tea and coffee consumption and myocardial infarction. A total of 340 subjects with well-documented myocardial infarction and 340 matched control subjects from the Boston Area Health Study provided dietary information. The investigators observed that individuals who drank more than one cup of tea per day had a 44% reduction in cardiovascular risk. In contrast, coffee consumption was not significantly associated with decreased or increased cardiovascular disease.

Additional support for a benefit of higher flavonoid intake is provided by studies relating red wine consumption to cardiovascular risk. There is extensive evidence that individuals who consume 1 or 2 drinks per day have reduced cardiovascular risk, compared with nondrinkers, and much of this benefit has been attributed to the direct effects of alcohol. However, there is evidence that red wine drinkers have additional benefit, and this observation has been interpreted to support a benefit of red wine polyphenols (18–20).

In addition to apparent benefits of flavonoid intake in the setting of primary prevention, one recent study suggested that flavonoid intake in the form of tea might have benefit among individuals with established cardiovascular disease. Mukamal et al (10) examined tea consumption in the Myocardial Infarction Onset Study, a prospective cohort study that examined 1900 patients admitted to community hospitals in the United States with acute myocardial infarction. During a follow-up period of 3.8 y, moderate and heavy tea drinkers had 31% and 39% reductions in cardiovascular risk, respectively, after adjustment for other risk factors.

In contrast to these studies suggesting a benefit, several studies demonstrated no relationship between flavonoid intake and cardiovascular risk (2, 5, 6, 12). It is notable that several of these neutral studies were performed in the United Kingdom, where tea consumption is relatively high (2, 6). Studies with relatively adjustment for known cardiovascular risk factors, compared with individuals in the lowest tertile.

Case-control studies also suggest that high flavonoid intake is beneficial. For example, Sesso et al (8) examined the relationship between tea and coffee consumption and myocardial infarction. A total of 340 subjects with well-documented myocardial infarction and 340 matched control subjects from the Boston Area Health Study provided dietary information. The investigators observed that individuals who drank more than one cup of tea per day had a 44% reduction in cardiovascular risk. In contrast, coffee consumption was not significantly associated with decreased or increased cardiovascular disease.

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well-nourished populations, such as US health professionals or college alumni, also tended to show less benefit of flavonoids (5, 12). In those studies, it is possible that confounding factors masked benefits of flavonoid consumption. For example, tea consumption is more common among individuals of low socioeconomic status in the United Kingdom, and such individuals are known to have increased risk for cardiovascular disease. Furthermore, baseline flavonoid intake may affect the results. If the population has a relatively high level of flavonoid consumption, then even subjects in the lowest quartile of consumption may be receiving the maximal benefits of flavonoid intake. This might provide an explanation for the lack of effect of tea observed in the United Kingdom, where consumption is high.

Overall, the evidence does suggest that higher consumption of flavonoids is associated with modest reduction of cardiovascular risk, despite the negative results of some studies. For tea, this conclusion is supported by a meta-analysis by Peters et al (17), which suggested an overall reduction in cardiovascular disease risk of ~11% with consumption of 3 cups of tea per day. A growing body of work has provided mechanistic information about how polyphenols might reduce cardiovascular disease, which provides additional support for a biological effect of polyphenols, rather than a simple association of polyphenol intake with a healthier lifestyle or other confounding factors.

**PATHOGENESIS OF CARDIOVASCULAR DISEASE EVENTS**

When considering how polyphenols might reduce cardiovascular risk, it is important to consider recent insights into the pathogenesis of atherosclerotic cardiovascular disease. Atherosclerosis is a chronic inflammatory disease that develops in lesion-prone regions of medium-sized arteries (21). Atherosclerotic lesions may be present and clinically silent for decades before becoming active and producing clinical events such as acute myocardial infarction, unstable angina, or sudden cardiac death. Such events are often caused by acute rupture or erosion of a vulnerable plaque, which exposes the highly thrombogenic subendothelium to flowing blood. The result is acute, platelet-rich, mural thrombosis that occludes or partially occludes the arterial lumen to produce infarction or ischemia. The precise mechanisms accounting for plaque vulnerability and rupture remain incompletely understood, but the available data suggest that local inflammation within the plaque, thinning of the fibrous cap, and accumulation of plaque lipid may contribute (22). Once plaque rupture occurs, the extent of thrombosis formation and acute changes in vascular tone may determine the extent of ischemia/infarction.

The development of a vulnerable plaque and the subsequent ischemic events represent a profound loss of vascular homeostasis. Recent studies focusing on vascular biological features provide a fruitful approach for development of novel treatments and prevention strategies for cardiovascular disease. In particular, there is great interest in antithrombotic therapies and therapies that influence the function of endothelial cells, which are key regulatory cells in the vessel wall. Relevant to the current review, there is increasing evidence that flavonoids may have beneficial effects on endothelial control of thrombosis, inflammation, and vascular tone. Flavonoids also have beneficial effects on platelets, which occlude the arterial lumen in the setting of acute coronary syndromes.

**ENDOTHELIAL FUNCTION AND CARDIOVASCULAR RISK**

The vascular endothelium plays a key role in the regulation of vascular homeostasis, and increasing evidence suggests that alterations in endothelial function contribute to the pathogenesis and clinical expression of cardiovascular disease (23). Endothelial cells regulate vascular homeostasis by producing factors that act locally in the vessel wall and lumen, and a key endothelial product is nitric oxide (24). Nitric oxide was first described as an endothelium-derived vasodilator, but it is now clear that nitric oxide regulates other important aspects of vascular homeostasis (25). For example, nitric oxide prevents adherence of leukocytes to the endothelial surface and inhibits expression of leukocyte adhesion molecules at the endothelial surface. Nitric oxide prevents platelet adhesion and platelet aggregation. Nitric oxide also inhibits vascular smooth muscle cell proliferation and alters expression of noncellular components that constitute the matrix of the vascular wall, making nitric oxide relevant to lesion formation, hypertrophy of the vessel wall, and vascular compliance. Therefore, endothelium-derived nitric oxide has important vasodilator, antiinflammatory, antithrombotic, and growth-suppressing properties that are relevant to all stages of atherosclerosis (23).

Other endothelium-derived products regulate vascular homeostasis, including other substances that influence vascular tone (eg, prostacyclin and endothelin), fibrinolytic factors (tissue plasminogen activator and plasminogen activator inhibitor-1), factors that affect coagulation (tissue factor, heparans, and von Willebrand factor), and proinflammatory factors (eg, adhesion molecules and inflammatory cytokines) (26). In general, loss of nitric oxide is paralleled by changes in these other regulatory mechanisms, leading to the development of a pathologic endothelial phenotype. These observations suggest that the state of the endothelium may be an indicator of vascular health and that examining endothelial vasomotor function may have clinical utility (23).

A common feature of otherwise diverse cardiovascular disease risk factors is their adverse effects on the endothelium. In this regard, dyslipidemia, hypertension, diabetes mellitus, smoking, aging, physical inactivity, systemic inflammation and infectious processes, hyperhomocysteinemia, and the postmenopausal state are all associated with endothelial dysfunction. Genetic and environmental factors might influence the effects of risk factors on endothelial function. Genetic variation in the activity of antioxidant enzymes or nitric oxide synthase might influence the effects of risk factors on endothelial function (27). Diet might also influence the effects of risk factors on endothelial function; therefore, polyphenol intake could be important in determining the risk for cardiovascular disease events.

Prospective studies have shown that endothelial dysfunction is associated with an increased risk of cardiovascular events (38 – 38). One study suggested that improved endothelial function after treatment of hypertension was associated with improved outcomes (35). Many interventions known to reduce cardiovascular disease risk have the ability to reverse endothelial dysfunction. For example, lipid-lowering therapy, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, smoking cessation, and exercise have all been shown to reverse endothelial dysfunction among patients with atherosclerosis or
STUDIES OF ANTIOXIDANTS AND ENDOTHELIAL DYSFUNCTION

With respect to the mechanism of endothelial dysfunction, there has recently been great interest in the importance of oxidative stress. These studies fit well with the recognition that oxidative stress contributes to atherogenesis (39). The importance of oxidative stress as a cause of endothelial dysfunction has prompted many investigations into the effects of antioxidants on endothelial function. For ascorbic acid in particular, there is extensive evidence that acute treatment reverses endothelial dysfunction in many disease states, including atherosclerosis, diabetes mellitus, hypertension, congestive heart failure, renal failure, hyperhomocysteinemia, hypercholesterolemia, and others (40).

Gokce et al (41) examined the effect of ascorbic acid on brachial artery flow-mediated dilation in a randomized, placebo-controlled, double-blind study of patients with coronary artery disease. Flow-mediated dilation reflects shear stress-mediated nitric oxide production by the endothelium (42). This physiologically relevant response is impaired in the setting of coronary risk factors and is correlated with abnormal responses in the coronary circulation (43); abnormal responses in the brachial artery predict future cardiovascular disease events among high- and low-risk patients (34–36, 38). In the study by Gokce et al (41), brachial artery flow-mediated dilation was impaired at baseline. Flow-mediated dilation improved 2 h after an acute 2-g dose of ascorbic acid, and the effect was sustained after 30 d of treatment with 500 mg/d. The responses were unchanged after acute and chronic treatment with placebo. Mechanistic in vitro studies suggested that the benefit of ascorbic acid may be attributable, in part, to increased activity of nitric oxide synthase resulting from stabilization of tetrahydrobiopterin, an essential cofactor for enzyme activity (44).

Other water-soluble antioxidant compounds have beneficial effects on endothelial function among patients with cardiovascular disease, including N-acetylcysteine (45, 46), glutathione (47), and the cysteine donor 1,2-oxothiazolidine-4-carboxylic acid (48). In contrast, the effects of lipid-soluble antioxidants on endothelium-dependent dilation among human subjects with atherosclerosis and cardiovascular risk factors have been relatively disappointing, although benefits have been reported for select, high-risk groups, including patients with type 1 diabetes mellitus (49) or multiple risk factors (50).

EFFECTS OF POLYPHENOLS ON ENDOTHELIAL FUNCTION

The extensive prior work demonstrating a beneficial effect of ascorbic acid on endothelial function prompted us to consider that other water-soluble antioxidants, such as flavonoids, might have beneficial effects on endothelial function. To explore this possibility, we investigated the effects of tea consumption on endothelial function. Tea contains an assortment of water-soluble antioxidant flavonoids, including catechins, quercetin, kaempferol, and other polyphenols, particularly thearubigin.

We examined the short-term and long-term effects of tea consumption on flow-mediated dilation in the brachial artery among 50 subjects with angiographically proven coronary artery disease, in a placebo-controlled, crossover study (51). Subjects taking antioxidant supplements were excluded, and subjects were asked to refrain from drinking tea and red wine during the study. All subjects were receiving prescribed medications for coronary artery disease, and 77% were undergoing lipid-lowering therapy. Short-term effects of tea were examined by measuring flow-mediated dilation before and 2 h after the subjects consumed 450 mL of freshly brewed black tea. Long-term effects were examined by measuring flow-mediated dilation again after the subjects had consumed 900 mL of black tea per day for 30 d. The study used water as a control beverage, and the beverage order was randomized.

Both short-term and long-term tea consumption improved endothelial function, whereas water consumption had no effect. There also were no effects of tea consumption on nitroglycerin-mediated dilation, baseline arterial diameter, or the extent of reactive hyperemia, which confirmed that tea consumption affected endothelial function, rather than the function of vascular smooth muscle or the stimulus for dilation. Flow-mediated dilation was not affected by an acute dose of caffeine, which suggested that the caffeine content of tea did not account for the results. Blood pressure, serum glucose concentrations, and serum lipid concentrations remained stable during tea consumption. Although catechins represent a relatively minor component of black tea, they are measurable in plasma. Our study demonstrated that total catechin amounts were increased ~20% after tea consumption; however, there was no relationship between changes in total catechin amounts and improvements in endothelial function.

Although tea contains flavonoid antioxidants, it remains unclear whether an antioxidant effect explains the observed benefits of tea. Our study demonstrated no effect of tea consumption on plasma antioxidant capacity (51). There also was no effect on plasma concentrations of F2-isoprostanes, markers of systemic lipid peroxidation, or 8-hydroxydeoxyguanosine, a marker of DNA oxidation. These findings are consistent with several other well-conducted studies that failed to demonstrate a reduction in markers of oxidative stress after tea consumption (52).

Several other studies demonstrated that flavonoid-containing beverages have beneficial effects on endothelial function. For example, Hodgson et al (53) examined the effect of tea consumption on brachial artery flow-mediated dilation among a group of otherwise healthy subjects with modest hypercholesterolemia. Those investigators observed that consumption of 5 cups of black tea per day for 5 wk led to improved flow-mediated dilation. Interestingly, tea consumption was also associated with an improvement in nitroglycerin-mediated dilation, which suggested that tea improved the bioactivity of endothelium-derived nitric oxide and/or had a beneficial effect on the function of vascular smooth muscle.

Other flavonoid-containing beverages, particularly grape products, have been shown to improve endothelial function. Stein et al (54) observed that consumption of grape juice for 14 d was associated with improved brachial artery flow-mediated dilation among 15 adults with angiographically proven coronary
artery disease. In that study, the susceptibility of LDL to ex vivo oxidation was reduced, which suggested an antioxidant effect. A second study from the same group also indicated beneficial effects of purple grape juice on endothelial function (55).

Agewall et al (56) observed an improvement in brachial artery flow-mediated dilation <1 h after consumption of dealcoholized red wine among healthy volunteers. In that study, consumption of wine (containing alcohol) was associated with vasodilation and increased blood flow but no observable increase in flow-mediated dilation. However, the effects of wine on baseline diameter and flow might have obscured an effect on endothelial function.

In a recent study, Heiss et al (57) examined the effects of cocoa on flow-mediated dilation. Among patients with at least one cardiovascular disease risk factor, impaired endothelial function was observed. Two hours after the patients consumed cocoa containing 176 mg/dL flavan-3-ols, the investigators observed a significant increase in flow-mediated dilation. They also observed increases in nitrosylated and nitrosated species in plasma, which suggested an increase in nitric oxide production.

### POLYPHENOLS AND PLATELET FUNCTION

Platelet aggregation plays a critical role in the pathogenesis of acute coronary syndromes, and there is extensive evidence that antiplatelet therapy reduces cardiovascular disease risk (58). An effect of polyphenols to reduce platelet activity could have a large impact on cardiovascular disease and might provide an important mechanistic explanation for the available epidemiologic data regarding polyphenols and cardiovascular disease.

Several basic studies demonstrated that flavonoids inhibit platelet aggregation (59, 60). Purple grape juice inhibited ex vivo platelet aggregation in whole blood (60). The direct clinical relevance of ex vivo platelet studies is unclear, and an important study by Demrow et al (59) examined the effects of grape juice on platelet function in vivo. Those investigators used the Folts model of unstable coronary stenosis, which involves the creation of endothelial injury and subocclusive stenosis in a dog coronary artery. In this model, transient platelet aggregation and release are reflected in cyclic variations in coronary blood flow; therefore, the model closely mimics a ruptured atherosclerotic plaque causing unstable angina. In this model, acute intragastric administration of red wine or grape juice was associated with marked reductions in cyclic flow variations, which indicates an important antiplatelet effect that is relevant to cardiovascular disease events (59).

Regarding the potential mechanisms of flavonoids on platelet function, Freedman et al (60) examined the effects of grape juice on platelet function. They observed that addition of grape juice to platelet ex vivo was associated with a reduction in platelet aggregation, a decrease in platelet production of superoxide anion, and an increase in platelet nitric oxide production. The beneficial effects appeared to be related to reduced activation of protein kinase C. Importantly, these findings were reproduced when the studies were repeated with platelets isolated from healthy volunteers who had consumed grape juice for 2 wk.

There are mixed data regarding the effects of tea consumption on platelet function. Animal studies of the effects of tea consumption on platelet aggregation in the Folts model suggested that tea may have benefits comparable to those of grape juice, although rather high doses of tea were required (JD Folts, personal communication, 2004). We examined the effects of short-term and long-term tea consumption on ex vivo platelet aggregation in response to ADP or thrombin-related activated peptide among patients with coronary artery disease (61). That study demonstrated no effect of tea consumption on platelet function, although the concurrent aspirin treatment could have influenced the results. Hodgson et al (62) observed that tea consumption reduced plasma concentrations of P-selectin, a marker of in vivo platelet aggregation. It is clear that additional studies will be required to define the effects of tea on platelet function.

### OTHER POLYPHENOLIC COMPOUNDS AND OTHER POTENTIAL MECHANISMS

Many other polyphenolic compounds have been reported to reduce cardiovascular disease risk and to have beneficial effects on endothelial and platelet function; a detailed discussion of these compounds is beyond the scope of the present review. For example, soy products, which are rich sources of isoflavones such as genistein and daidzein, have been reported to improve endothelial function, possibly through an effect on the α estrogen receptor (63).

There is great interest currently in the importance of systemic inflammation as a pathogenic mechanism of cardiovascular risk (64). Importantly, there is growing evidence that polyphenolic compounds may have antiinflammatory effects. For example, the grape and wine component resveratrol inhibits adhesion molecule expression and monocyte adhesion in vitro (65). Additional study of the antiinflammatory effects of polyphenols is clearly warranted.

### CONCLUSIONS

This article has reviewed epidemiologic and mechanistic studies that suggest that polyphenols have beneficial effects on the cardiovascular system and reduce the risk of cardiovascular disease. It should be emphasized that some of the epidemiologic data are conflicting, and this review considered some possible explanations for the reported negative findings for certain well-nourished populations and populations with high flavonoid intake overall. Improved endothelial function, antiplatelet effects, and antiinflammatory effects are among the important mechanisms to be considered for the observed benefits. Overall, the findings of available studies fit well with the recommendations of the American Heart Association that Americans should increase their consumption of fruits and vegetables and other foods with high polyphenol contents (66).

### REFERENCES


Cocoa antioxidants and cardiovascular health

Carl L Keen, Roberta R Holt, Patricia I Oteiza, César G Fraga, and Harold H Schmitz

ABSTRACT
An increasing body of epidemiologic evidence supports the concept that diets rich in fruits and vegetables promote health and attenuate, or delay, the onset of various diseases. Epidemiologic data support the idea that these health benefits are causally linked to the consumption of certain flavonoids present in fruit and vegetables. In the context of cardiovascular health, a particular group of flavonoids, namely, the flavan-3-ols (flavanols), has received attention. Flavanol-rich, plant-derived foods and beverages include wine, tea, and various fruits and berries, as well as cocoa and cocoa products. Numerous dietary intervention studies in humans and animals indicate that flavanol-rich foods and beverages might exert cardioprotective effects with respect to vascular function and platelet reactivity. This review discusses the bioactivity of flavanols in the context of cardiovascular health, with respect to their bioavailability, their antioxidant properties, and their vascular effects.

KEY WORDS Antioxidants, cardiovascular health, chocolate, cocoa, flavanols, platelets, procyanidins

INTRODUCTION
An increasing body of epidemiologic evidence supports the concept that diets rich in fruits and vegetables promote health and attenuate, or delay, the onset of various diseases, including cardiovascular disease, cancer, and certain neurodegenerative disorders (ref 1 and references therein). Furthermore, the epidemiologic data support the idea that these health benefits can be linked, in part, to the presence of certain flavonoids in fruits and vegetables (ref 2 and references therein). In the context of cardiovascular health, a particular group of flavonoids, namely, the flavan-3-ols (flavanols), has recently received attention. Flavanol-rich, plant-derived foods and beverages include wine, tea, and various fruits and berries, as well as cocoa and cocoa products, in which flavanols can be present as either the monomers (-)-epicatechin and (+)-catechin or oligomers of epicatechin and/or catechin (procyanidins) (3). Numerous dietary intervention studies with humans and experimental animals indicate that flavanol-rich foods and beverages can exert cardioprotective effects with respect to vascular function and platelet reactivity (4–8). Although data from the aforementioned studies are well correlated with epidemiologic findings and thus strongly support flavanols as cardioprotective agents, the mechanisms underlying these flavanol-mediated biological effects in vivo are not well defined. On the basis of several in vitro studies, however, it can be speculated that mechanisms contributing to the biological effects of flavanols may include their antioxidant effects, their ability to modulate certain cell signaling pathways and gene expression, and their ability to influence cell membrane properties and receptor function. This review discusses the bioactivity of flavanols in the context of their ability to affect cardiovascular health. Attention is focused on flavanol bioavailability, the antioxidant properties of flavanols, and the potential direct vascular effects of these nutrients.

FLAVANOL AND PROCYANIDIN BIOAVAILABILITY
In discussions of the biological activity of flavanols or procyanidins, several factors must be considered, such as whether they can be absorbed, their tissue and cellular distributions after absorption, and their bioactive forms. Flavanols are distinct from other flavonoid classes; instead of appearing as glycosides, flavanols are present in the aglycone form, as oligomers, or esterified with gallic acid (9). Although it was initially thought that procyanidins were degraded under the acidic conditions of the stomach (10), data from human subjects showed that flavanols and procyanidins are stable during gastric transit (11). Once in the mesenteric circulation, flavanols exist predominately in a conjugated form. The flavanols are absorbed from the jejunal lumen into the epithelial cell layer, where they are methylated and glucuronidated (12, 13). In the liver, additional glucuronidation and methylation, as well as sulfation, can take place (12, 13). In addition to being present in the plasma and urine of experimental animals and human subjects (14–16), flavanol conjugates have been found in rat bile (16) and brain (17). It has been reported that colonic microflora can degrade the flavan structure of flavonoids to form simple phenolic and ring-fission metabolites that may be physiologically relevant (18, 19). Among human subjects, increased urinary excretion of 4 phenolic acids was found 9–48 h after cocoa consumption (20). These phenolic acids (m-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid, m-hydroxybenzoic acid, and m-hydroxymethylacetic acid) are possible products of colonic microbial degradation of the procyanidins (20); therefore, products of colonic metabolism must be considered in examinations of the potential chronic biological effects of cocoa and chocolate, as well as other flavanol- and procyanidin-rich foods.

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Among humans, plasma concentrations of nonmethylated epicatechin in the micromolar range can be found as early as 1 h after cocoa consumption (14, 21, 22), with the major metabolites being epicatechin-7-β-d-glucuronide and epicatechin-7-sulfate. Methylated epicatechin forms, such as 3’-O-methyl(epicatechin), can also be found in the plasma in the micromolar and range, and, like the rest of the metabolites, are rapidly excreted (14). There is some evidence that certain flavanols are better absorbed than others. Lee et al (23) reported that, whereas epigallocatechin and epigallocatechin-3-gallate were present at similar concentrations in a green tea drink, the plasma epigallocatechin concentration was 2–3 times greater than the epigallocatechin-3-gallate concentration after tea consumption. Similar results have been reported for cocoa. After human subjects were given a cocoa beverage containing epicatechin and catechin in a 1:1 ratio, peak plasma catechin concentrations were <10% of the peak epicatechin concentrations (0.16 and 5.93 µmol/L, respectively) (21).

Part of this difference in plasma concentrations between epicatechin and catechin may be attributable to dimer cleavage (15, 24). Although several research groups have examined the bioavailability of the monomeric flavanols, there is limited information on the metabolism of the procyanidins. Dimer B2 [epicatechin-(4β-8)-epicatechin] and dimer B5 [epicatechin-(4β-6)-epicatechin] have been detected in the nanomolar range in the plasma of humans and rats (15, 21, 22, 25), whereas other procyanidins, such as dimer B3 [catechin-(4α-8)-catechin] (26) and trimer C2 [catechin-(4α-8)-catechin-(4α-8)-catechin] (26), could not be detected. It is important to note that the oligomers found in the plasma are those consisting of epicatechin and not catechin subunits. Therefore, the observed differences in plasma concentrations between epicatechin and catechin, and their oligomers, might be attributable to stereochemical differences, which result in differences in hydrophobicity and some biological properties, such as antioxidant activity (27–29).

Cocoa and chocolate represent food and beverage products that have varying concentrations of flavanoids, as a result of many different factors. As with most plants, genetic and agronomic factors can markedly influence the contents of phytochemicals available at the time of harvest. Postharvest handling plays a critical role at this juncture, because most cacao beans undergo some fermentation steps, which subject flavanoids in the cacao to heat and acidic conditions (29). Subsequent processing steps, such as roasting and alkali treatment, can also reduce the flavonoid contents. Finally, the recipe for the finished food or beverage product determines the amount of a given cocoa (and flavanoid) added. Depending on harvesting and processing procedures, as much as 90% of the flavonoids can be lost during processing (30, 31).

This illustrates the potential effects of food processing on the flavonoid contents of foods. Similarly, there can be significant food matrix effects on the bioavailability and potential biological activity of cocoa flavanols and procyanidins. A recent study suggested that milk proteins reduced the antioxidant activity of milk chocolate, compared with dark chocolate (32). Although the protein-binding ability of the flavanols and procyanidins is well known (33, 34), studies of flavanol consumption from tea showed large (35, 36) or no (37, 38) effects of milk addition on antioxidant activity and flavanol bioavailability. In a recent study, no differences in either antioxidant capacity or epicatechin bioavailability were observed when cocoa was provided with or without milk, under isocaloric and islipidemic conditions, to healthy human subjects (39). Therefore, observed differences in antioxidant activity and bioavailability between dark chocolate and milk chocolate (32) were the result of the food matrix altering the kinetics of absorption and were likely not attributable to flavanol-milk protein interactions.

### FLAVANOLS AND PROCYANIDINS FROM COCOA AS ANTIOXIDANTS

Cocoa, cocoa extracts, and purified cocoa flavanols and procyanidins exert strong antioxidant effects in vitro. The antioxidant properties of flavanols are based in part on their structural characteristics, including the hydroxylation of the basic flavan ring system, especially 3’,4’-dihydroxylation of the B-ring (catechol structure), the oligomer chain length, and the stereochemical features of the molecule (40). These structural characteristics of flavanols represent the molecular basis for both their hydrogen-donating (radical-scavenging) properties and their metal-chelating antioxidant properties. For example, cocoa and purified cocoa flavanols and procyanidins have been reported to attenuate the copper-mediated and endothelial cell-mediated oxidation of LDL (41, 42), to reduce the production of reactive oxygen species by activated leukocytes (43), to protect against erythrocyte hemolysis (25, 44), and to inhibit ultraviolet C-induced DNA oxidation (45). In the latter case, the cocoa flavanols that were tested were as effective (on a molar basis) as ascorbate, α-tocopherol, and glutathione. Interestingly, cocoa powder and cocoa extracts have been shown to exhibit greater antioxidant capacity than many other flavanol-rich foods and food extracts, such as green and black tea, red wine, blueberry, garlic, and strawberry (41, 46).

The aforementioned in vitro data have been shown to translate, at least in part, into in vivo model systems. Among healthy human subjects, inhibition of LDL oxidation was reported within 2 h after the consumption of a flavanol-rich cocoa product (47). After rats were provided an oral dose of cocoa powder, the rates of both copper and 2,2’-azo-bis(2-aminopropane) dihydrochloride (AAPH)-induced LDL oxidation were significantly reduced (48). Similarly, long-term feeding studies with a flavanol-rich cocoa showed an increase in total plasma antioxidant capacity (49) and a reduction in the susceptibility of LDL to ex vivo oxidation (49, 50). In the aforementioned studies, no differences were observed between the control and cocoa-fed groups with respect to total plasma cholesterol, triacylglycerol, LDL, or HDL concentrations. In rats, the chronic consumption of diets containing 2% cocoa powder, providing 1.57 mg/g diet of flavanols and procyanidins, was associated with reduced DNA and glutathione oxidation (51). Diabetes mellitus-induced cataracts and ex vivo lipid peroxide formation were decreased in rats given cocoa liquor (52). Among human subjects, the consumption of a flavonoid-rich chocolate increased plasma antioxidant capacity and reduced the amounts of plasma 2-thiobarbituric acid-reactive substances, in a dose-dependent manner (53). Although the precise mechanisms underlying the antioxidant effects of flavanoids have yet to be identified, the research reported to date suggests that consumption of flavanols and procyanidins in the diet can significantly augment the oxidative defense system.

What are the possible mechanisms that contribute to the antioxidant protection of flavanols? For the flavanols present in cocoa (epicatechin and catechin), antioxidant activity is attributed to the presence of a catechol group on the B ring, which can trap free radicals and chelate redox-active metals (9, 40). Therefore, a reasonable hypothesis would be that antioxidant activity...
increases with increasing oligomer chain length. Consistent with this, Lotito et al (54) observed that, in a model of liposome oxidation, the antioxidant capacity of cocoa procyanidins was influenced by oligomer chain length and the nature of the oxidant. When AAPH (a water-soluble radical generator) or ultraviolet C light was used as a radical initiator, dose-dependent protection was observed; however, this protection was not dependent on oligomer chain length according to monomeric equivalents, which allows comparison of equivalent numbers of catechol groups. In contrast, when 2,2'-azo-bis(2,4-dimethylvaleronitrile) (a lipid-soluble radical generator) was used as an initiator, an increase in chain length was associated with increased protection. Finally, an inverse association of chain length with protection was observed with the use of ferrous ascorbate as the initiator (54). Consistent with this, Steinberg et al (22) observed no differences in the capacity of monomers, dimers, pentamers, and hexamers to inhibit LDL oxidation when AAPH or copper was used as an oxidizing agent. However, Verstraeten et al (55) observed that procyanidin chain length was a determinant of the capacity to protect liposomes against 2,2'-azo-bis(2,4-dimethylvaleronitrile)-induced oxidation and against membrane disruption induced by the detergent Triton X-100. The accumulation of procyanidins at the liposome surface, through hydrogen bonding with the polar head group of phospholipids, prevented access of deleterious molecules to the hydrophobic core of the bilayer (55).

Taken together, these studies suggest that the flavanols and procyanidins have different interactions with biological membranes. Schroeder et al (28) demonstrated that epicatechin is amphiphilic in nature, with an observed partition coefficient in an octanol-buffer system of 1.45. In the same study, catechin was more lipophilic (partition coefficient of 2.92) than epicatechin, and compounds containing gallic acid and glucosyl residues, such as epigallocatechin-3-gallate, rutin, and α-glucosyl-rutin, were hydrophilic in nature. Similar partition coefficient values were observed for catechin, morin, and taxifolin (partition coefficients of 2.92, 2.53, and 2.02, respectively), which suggests that it is the difference in stereochemistry at C3 between epicatechin and catechin that affects hydrophobicity. The authors also observed uptake of epicatechin in cell lysates of murine aortic endothelial cells after 30 min of incubation (28). In a separate study, catechin and epicatechin dose-dependently accumulated to similar extents in the whole-cell fraction of Jurkat cells, and epicatechin, but not catechin, could be detected in the nuclear fraction. Dimer B2 was also found in both the whole-cell and nuclear fractions, although to a lesser extent than epicatechin (56). Therefore, in contrast to longer-chain oligomers, the flavanol monomers and dimers (particularly those with epicatechin subunits) appear to be able to diffuse across the membrane and into the cell. As discussed below, the ability of the smaller oligomers to diffuse into the cell is important, because cocoa products and isolated flavanols and procyanidins have been observed to affect enzymes andsignaling cascades.

VASCULAR EFFECTS OF FLAVANOL-RICH COCOA AND CHOCOLATE

Other mechanisms

Although flavanol-rich cocoa and chocolate have the potential to augment an individual’s antioxidant defense system, there are other cellular mechanisms through which these flavanol-rich foods can affect cardiovascular health. Inflammation, platelet aggregation, and nitric oxide (NO)-mediated endothelial changes are additional factors that can be influenced by flavanols.

As a broad concept that applies to this discussion, it is important to recognize the interaction between reactive oxygen species and reactive nitrogen species that occurs in vivo, which results in the formation of peroxynitrite (a powerful oxidant) and the consumption of NO (decreasing vasorelaxation) (57). As discussed above, flavanols and procyanidins can trap reactive oxygen species, thus acting as effective protectors against peroxynitrite-dependent oxidation and nitration reactions (58).

Inflammation

Atherosclerosis and heart failure, as well as risk factors such as hypertension and hypercholesterolemia, can activate several proinflammatory enzyme systems, such as xanthine oxidase, NADH/NADPH oxidase, and myeloperoxidase (59). Once activated, these enzymes produce reactive oxygen species and other radicals that, as indicated above, can modify NO availability and LDL and contribute to endothelial dysfunction (59). Flavanol-rich cocoa liquor has been shown to stimulate NO production and to significantly reduce the activities of xanthine oxidase and myeloperoxidase after ethanol-induced oxidative stress (60). In addition, cocoa flavanols and procyanidins may modulate other mediators of inflammation. For example, there is emerging evidence that flavanols and procyanidins can suppress the production of the proinflammatory cytokines interleukin (IL)-1β and IL-2 (43, 61) in peripheral blood mononuclear cells, enhance the production of the antinflammatory cytokine IL-4 (62), suppress 15-lipoxygenase activity (63), and beneficially modulate transforming growth factor-β, and tumor necrosis factor-α concentrations (64) in peripheral blood mononuclear cells. Catechins have also been reported to suppress microvascular endothelial cell production of IL-8 (65, 66), a potent chemoattractant in the initiation and progression of atherosclerosis (67).

Rel/NF-κB transcription factors are activated by multiple signals and regulate the expression of numerous genes involved in inflammation, cell proliferation and survival, and stress and immune responses (68). NF-κB regulates gene transcription of cytokines and adhesion molecules involved in the onset and progression of atherosclerosis. Activated NF-κB is present in macrophages, vascular smooth muscle cells, and endothelial cells of atherosclerotic lesions (69, 70). In rats, NF-κB is also activated in vascular smooth muscle cells after arterial injury (71). Significantly, the use of a cis-element decoy for NF-κB, a synthetic double-stranded DNA that binds NF-κB with high affinity and inhibits NF-κB-driven gene expression, prevents the vascular hyperplasia that occurs after rat carotid artery injury (72).

Epicatechin, catechin, and an isolated fraction of B-type dimers (B2 and B5) were recently shown to regulate NF-κB (56). This regulation occurs at multiple cell levels, in the early cytotoxic events in the NF-κB activation cascade (ie, modulation of oxidant concentrations, IκB kinase activation, and subsequent IκBα phosphorylation) and at later stages (inhibition of NF-κB binding to its consensus DNA sequence). Procyanidins have been observed to modulate the expression of the NF-κB-dependent IL-2 and IL-1β (43, 61). As evidence that epicatechin, catechin, and the B-type dimers isolated from cocoa can inhibit NF-κB-driven gene transcription, Mackenzie et al (56) demonstrated decreased IL-2 expression in Jurkat cells.
Some of the effects of epicatechin, catechin, and the B-type dimers on NF-κB can be attributed to the antioxidant capacity of these compounds. There is evidence indicating that NF-κB is a transcription factor sensitive to oxidant stimuli and to changes in the cellular thiol redox state (73, 74). In Jurkat cells, phorbol-12-myristate 13-acetate induced an increase in cell oxidant concentrations that was inhibited by epicatechin, catechin, and the B-type dimers (56). Similarly, in RAW 264.7 macrophages stimulated with IFN-γ, the flavanols and dimers inhibited NO production and NF-κB-dependent transcriptional activity (75).

Platelets

Given the role of platelets in the development and manifestation of acute myocardial infarction, stroke, and venous thromboembolism, several antiplatelet strategies have been developed to prevent secondary events. Several studies suggest that, in addition to providing antioxidant vitamins, certain fruits and vegetables may provide protection against thrombosis because of their flavanol contents. Cocoa was shown to reduce ADP/collagen- and adrenaline/collagen-activated, platelet-related, primary hemostasis within hours after subjects consumed high (897 mg) or moderate (220 mg) amounts of cocoa flavanols (76–78). These antiaggregatory effects observed with cocoa were shown to be attributable, in part, to a reduction in the ADP- and adrenaline-induced expression of the activated conformation of the GPIIa/IIIb surface protein (76, 78). In addition, cocoa was able to reduce GPIIa/IIIb expression to an extent that was only slightly less than that achieved with low-dose aspirin (81 mg) (78). The effects observed in the aforementioned short-term studies could also be extended to a 4-wk study, during which subjects consumed moderate amounts of cocoa flavanols. As a result, decreases in P-selectin expression, ADP-induced platelet aggregation, and platelet volume (marker of lowered activation status) were observed (5).

It is possible that flavanols may mediate their activity through antioxidant and NO-related mechanisms, and such mechanisms can be implicated in platelet function. Superoxide anion is known to enhance platelet aggregation and can bind to NO to form peroxynitrite. Freedman et al (8) observed that purple grape juice consumption reduced platelet superoxide release, with a corresponding increase in platelet NO production and a reduction in platelet aggregation. In addition, catechin was shown to reduce platelet aggregation and hydrogen peroxide production in vitro (79). These studies suggest that flavanols may exert their antioxidant effects through their well-described antioxidant activities, although it should be emphasized that flavanols may act through nonantioxidant mechanisms. For example, purple grape juice has been reported to reduce platelet protein kinase C activity (8), whereas dimer B2 has been shown to inhibit platelet thromboxane production (80). In addition, cocoa flavanols and procyanidins have been reported to inhibit human platelet 12-lipoxigenase (63) and 5-lipoxigenase (81). Inhibition of 5- and 12-lipoxigenase could partly explain the observations of Schramm et al (82) and Holt et al (77) that the ratio of plasma prostacyclin and leukotriene concentrations increases among human subjects after the consumption of flavanol-rich chocolate.

Endothelium

The vascular endothelium regulates hemostasis through maintenance of vasomotor tone and through its influence on platelet function and leukocyte adherence. Under normal physiologic conditions, several mediators are released from the endothelium, such as endothelin, prostacyclin, leukotrienes, NO, and adenosine (83). Shear stress, ischemia and reperfusion, inflammation, and disease states, such as atherosclerosis, diabetes mellitus, and hypertension, can disrupt endothelial function, which is associated with alterations in endothelium-derived regulatory mediators, an inability to regulate vascular tone, and an overall shift toward the prothrombotic state. It is possible that flavanols, by functioning as antioxidants in addition to modulating prostacyclin and leukotriene concentrations (82), can improve endothelial function through the prevention and possible reduction of oxidative damage. However, other mechanisms that have yet to be elucidated may also be involved.

CONCLUSIONS

There is now a large body of information that supports the idea that cocoa flavanols and procyanidins have the ability to act as in vivo antioxidants. These nutrients have been shown to affect numerous intracellular signaling cascades and to influence the cardiovascular system by enhancing vascular function and decreasing platelet reactivity. Several in vivo studies have provided strong support for the hypothesis that the consumption of flavanol-rich foods, such as certain cacao and chocolates, may be associated with reduced risk for vascular disease. Significantly, in vitro studies with highly purified flavanols and procyanidins support the hypothesis that many of the biological effects observed with flavonoid-rich foods can be directly attributed to the flavonoids. A number of important questions remain, however. For example, there is little information on the extent to which flavonoids interact with other nutrients in the diet before and after absorption. There is also limited information on the intracellular metabolism of these compounds and on the bioactivity of the different metabolites. Another area in which we have a dearth of information concerns the acute and chronic effects of dietary flavonoids. Although several short-term clinical trials have been reported, the health effects of these nutrients will best be determined from long-term, randomized, clinical trials.

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Cocoa polyphenols and inflammatory mediators

Helmut Sies, Tankred Schewe, Christian Heiss, and Malte Kelm

ABSTRACT

Cocoa products are sources of flavan-3-ols, which have attracted interest regarding cardiovascular health. This review provides a survey of our research on the effects of cocoa polyphenols on leukotriene and nitric oxide (NO) metabolism and on myeloperoxidase-induced modification of LDL. Because intake of flavonoid-rich chocolate by human subjects was reported to decrease the plasma concentrations of proinflammatory cysteinyl leukotrienes, we assessed whether cocoa polyphenols inhibited human 5-lipoxygenase, the key enzyme of leukotriene synthesis. (−)-Epicatechin and other cocoa flavan-3-ols proved to be inhibitory at the enzyme level. This action may confer antileukotriene action in vivo. In a double-blind crossover study, 20 individuals at risk for cardiovascular diseases received cocoa beverages with high or low contents of flavan-3-ols. NO-dependent, flow-mediated dilatation of the brachial artery and concentrations of nitroso compounds in plasma were measured, and it was shown that ingestion of the high-flavanol cocoa drink but not the low-flavanol cocoa drink significantly increased plasma concentrations of nitroso compounds and flow-mediated dilatation of the brachial artery. Therefore, ingested flavonoids may reverse endothelial dysfunction through enhancement of NO bioactivity. Oxidative modification of LDL appears to be crucial for atherogenesis, and one of the mediators is the proinflammatory proatherogenic enzyme myeloperoxidase. Micromolar concentrations of (−)-epicatechin or other flavonoids were found to suppress lipid peroxidation in LDL induced by myeloperoxidase in the presence of physiologically relevant concentrations of nitrite, an NO metabolite. Adverse effects of NO metabolites, such as nitrite and peroxynitrite, were thus attenuated. Am J Clin Nutr 2005;81(suppl):304S–12S.

KEY WORDS Antioxidants, arachidonic acid, atherogenesis, chocolate, cocoa, flavan-3-ols, flavonoids, flow-mediated dilatation, inflammation, leukotrienes, lipoxygenase, LDL, myeloperoxidase, nitrite, nitrosothiols, peroxynitrite, procyanidins

INTRODUCTION

Flavonoids constitute a group of natural compounds that occur in fruits and vegetables, wine and tea, and also chocolate and other cocoa products. Recently, flavonoids have attracted increasing interest from nutritional biochemists. First, these polyphenols exert potent antioxidant actions in numerous in vitro systems (1–3). Second, the daily dietary intake of flavonoids and similar polyphenols exceeds that of antioxidative vitamins and provitamins. In epidemiologic studies, increased intake of flavonoids was associated with reduced risk of major cardiac events (4–7). The presumed beneficial effects of flavonoids are mainly ascribed to their inherent capacity to scavenge reactive oxygen and nitrogen species, thus counteracting conditions of oxidative stress that accompany disorders such as coronary artery disease and other vascular diseases, stroke, inflammatory diseases, and cancer. However, several nonantioxidant effects of flavonoids are also being considered (1–3).

For a fuller understanding of the protective actions of flavonoids against oxidative stress, the effects of flavonoids on oxidant enzymes such as lipoxygenases and myeloperoxidase (MPO) must be elucidated. Lipoxygenases are involved in arachidonic acid metabolism leading to several inflammatory mediators, including (among others) leukotrienes, conjugated hydroxyeicosatetraenoic acids (HETEs) (predominately 5-HETE, 12-HETE, and 15-HETE), heparin, and lipoxins (8–10), in addition to cyclooxygenase-mediated eicosanoids such as prostaglandins, prostacyclin, and thromboxanes. Non–heme iron-containing lipoxigenases and heme-containing cyclooxygenases are dioxygenases that primarily attack one of the bis-allylic methylenes present in arachidonic acid or other polyenoic fatty acids. This initial reaction step is shared with nonenzymatic lipid peroxidation and yields analogous compounds (hydroperoxyeicosatetraenoic acids and isoprostanes) but, unlike in the nonenzymatic route, with positional and stereochemical specificity. Oxygenating enzymes involved in eicosanoid syntheses may also generate deleterious, fatty acid-derived, free radicals and reactive oxygen species through side reactions, which could be blunted by flavonoids and other dietary polyphenols.

The interactions of dietary flavonoids with arachidonic acid metabolism have not been fully elucidated. It was reported that intake of flavonoid-rich chocolate by volunteers caused a significant decrease in the ratio of the concentrations of the plasma metabolites of cysteinyl leukotrienes and prostacyclin (prostaglandin I2) (11). These changes were paralleled by significant increases in the plasma concentrations of (−)-epicatechin and its metabolites, which suggested that the observed beneficial effects of chocolate on arachidonic acid metabolism were attributable to...
its high concentrations of (−)-epicatechin and its oligomers, the procyanidins (12). The molecular targets of these flavanols remain to be addressed, however.

We studied the interactions of the flavonoids of cocoa with selected enzymes of arachidonic acid metabolism. 5-Lipoxygenase, the key enzyme in the synthesis of proinflammatory leukotrienes (9), was of particular interest. Other mammalian lipoxygenases also merit some attention. For example, the subfamily of reticulocyte-type 12/15-lipoxygenases (eg, human and rabbit 15-lipoxygenase-1) has been proposed to be involved in early stages of atherogenesis and may be a potential target for compounds that are protective for the cardiovascular system (13, 14). Flavonoids were reported to ameliorate progression of atherosclerosis in apolipoprotein E–deficient mice (15). For the same reason, we also studied the interactions of cocoa flavonoids with MPO, another prooxidant enzyme that is thought to be involved in atherosclerosis and inflammation (16, 17).

Vascularprotective actions of dietary flavonoids, as evidenced by improvement of endothelial function, were noted after intake of flavonoid-containing beverages such as black tea or red wine (18, 19). These effects are apparently attributable not only to scavenging of free radicals and suppression of proinflammatory mediators but also to enhancement of bioactive nitric oxide (NO), an antiinflammatory mediator and vasodilator with concomitant antiplatelet activity (20–22). The bioavailability of NO is determined by both synthesis via various isoforms of NO synthase and conversion to nitrite, nitrate, or other products. The various NO metabolites differ considerably with respect to their biological activities (23–25). Although the bioactivity of NO is preserved in S-nitrosothiols, N-nitroso compounds, and nitrosohemoglobin [collectively referred to as organic nitroso compounds (RNO)] (24), which contribute to the circulating NO pool in human blood (25), nitrite and peroxynitrite may exert predominantly adverse effects. For example, nitrite is a known substrate for MPO (26), and the MPO/nitrite system is involved in the modification of LDL, rendering it atherogenic (27). To examine the improvement of NO bioactivity by cocoa polyphenols in vivo, we compared the effects of high-flavanol and low-flavanol cocoa beverages on plasma nitroso compounds and on flow-mediated dilation (FMD) of the brachial artery among individuals at risk for cardiovascular diseases. Our data from both in vitro and in vivo studies substantiate the purported beneficial actions of cocoa polyphenols on the human cardiovascular system.

INHIBITION OF MAMMALIAN LIPOXGENASES

To elucidate whether the reported decrease in the plasma concentrations of cysteinyl leukotrienes with intake of procyanidin-rich chocolate (11) could be attributable to direct inhibition of arachidonate 5-lipoxygenase by flavan-3-ols at the enzyme level, we investigated the effects of flavonoids occurring in cocoa on the reaction of isolated recombinant human 5-lipoxygenase with arachidonic acid (28). As shown in Figure 1, the formation of the major primary product, 5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HETE), was inhibited by (−)-epicatechin to completeness in a dose-dependent manner, with a concentration of half-inhibition (IC_{50}) of 22 μmol/L. The formation of hydrolysis...
products of 5,6-leukotriene A₄ (diastereomers of 5,12- and 5,6-
dihydroxyeicosatetraenoic acids) was inhibited with comparable
potency (28). It follows from these observations that (−)-
epicatechin inhibits both the dioxygenase and leukotriene A₄
synthase activities of 5-lipoxygenase, ie, the first 2 consecutive
steps of the conversion of arachidonic acid into various pro-
flammatory leukotrienes, which are catalyzed by the same en-
zyme (9, 10). The dual effects of the flavan-3-ol on the
5-lipoxygenase-catalyzed reactions indicate direct blockage of
the active site of the enzyme. An involvement of 5-lipoxygenase-
activating protein in the inhibitory action appears to be ruled out,
because the activity of 5-lipoxygenase is dependent on
5-lipoxygenase-activating protein only in intact cells or in re-
constituted systems (30) and not in a membrane-free system, as
used here.

Mammalian lipoxygenases constitute a family of closely re-
lated enzymes that share a common basic catalytic mechanism
and similar active site structures but differ genetically and with
respect to the positional specificity of the dioxygenation of ara-
chidonic acid and other enzymatic characteristics (31–33).
Therefore, the question of whether flavan-3-ols are general in-
hbitors of mammalian lipoxygenases, irrespective of genetic
subfamily, reaction specificity, and biological function, arose.
We demonstrated that purified 15-lipoxygenase-1 from rabbit
reticulocytes was inhibited by (−)-epicatechin and related
flavan-3-ols, as well as by the flavonol quercetin (34) and even
more effectively by the flavone luteolin (35). The inhibitory
effects occurred in every case, irrespective of whether arach-
didonic acid or linoleic acid was used as substrate or whether
conjugated diene formation, oxygen consumption, or formation
of a specific reaction product (eg, 15S-hydro(pero)xy-
5Z,8Z,11Z,13E-eicosatetraenoic acid from arachidonic acid)
was measured. The mode of the inhibitory action of flavonoids on
this enzyme and the structure-activity relationships were studied
in more detail and revealed complexity involving both reversible
and irreversible processes (35). The inhibitory effects of (−)-
epicatechin and procyanidins were also observed with human
15-lipoxygenase-1 and porcine leukocyte 12-lipoxygenase (34),
which belong to the same subfamily (reticulocyte-type 12/15-
lipoxygenases)

Recombinant human platelet 12-lipoxygenase, a representa-
tive of another subfamily of mammalian lipoxygenases, also was
inhibited by (−)-epicatechin (Figure 2). Therefore, we conclude
that this compound and related flavan-3-ols are general inhibitors
of mammalian lipoxygenases (Table 1).

A large proportion of the flavonoids in cocoa products are
present as oligomeric procyanidins. Therefore, we also tested the
effects of procyanidin fractions, isolated from the seeds of the
cocoa tree Theobroma cacao according to oligomer size, on both
recombinant human 5-lipoxygenase and rabbit reticulocyte
15-lipoxygenase-1 (Figure 3). It should be emphasized that in these
studies we applied identical amounts by weight, so that the molar
concentrations decreased with increasing oligomer size, on the
basis of the assumption that each epicatechin subunit of the
procyanidin molecule might contain the relevant structural fea-
tures required for inhibitory capacity (eg, the 5 hydroxyl groups).
With 5-lipoxygenase, only the fractions of small procyanidins
(dimer through pentamer) revealed inhibitory potencies similar
to those of the monomer, which continuously decreased with
increasing molecular mass, whereas the larger procyanidin frac-
tions produced only weak effects (if any). A quite different pat-
tern was noted for 15-lipoxygenase-1. The inhibitory potency
first decreased from monomer to tetramer, reaching a minimum,
but then increased with increasing oligomer size, with the
decamer fraction being most potent (IC₅₀: 0.8 μmol/L). Unlike
(−)-epicatechin, the procyanidin fractions from cocoa also in-
hibited the activity of soybean lipoxygenase L-1, with potencies
that continuously increased with increasing oligomer size (34).

Other monomeric flavan-3-ols also inhibited rabbit 15-
lipoxygenase-1. (−)-Catechin did not reveal any significant dif-
fERENCE, compared with its (−)-epimer. In contrast, the tea
flavan-3-ol (−)-epigallocatechin gallate was a much more potent
inhibitor of both 15-lipoxygenase-1 [IC₅₀: ~4 μmol/L (34)] and
5-lipoxygenase [IC₅₀: ~3 μmol/L (28)]. These higher potencies
are presumably attributable to the gallic acid moiety, because
aliphatic alkyl gallates were reported also to be strong 5- and
15-lipoxygenase inhibitors (38, 39). Greater inhibitory potency,
compared with (−)-epicatechin, was observed also for the fla-
vonol quercetin, with IC₅₀ values of ~4 μmol/L for 15-
lipoxygenase-1 (34) and ~0.6 μmol/L for 5-lipoxygenase (28).

**FIGURE 2.** Effects of (−)-epicatechin and the procyanidin decamer fraction isolated from the seeds of *Theobroma cacao* (36) on the reaction of recombinant human platelet 12-lipoxygenase with arachidonic acid. Oxygen consumption was measured oxygenographically (Oxygrometer 781; Strathkelvin Instruments, Glasgow, United Kingdom), in 0.1 mol/L air-equilibrated potas-
sium phosphate (pH 7.4) containing 0.1 mmol/L diethylenetriamine pen-
taacetic acid, at 20 °C. Recombinant human 12-lipoxygenase (Calbiochem,
Bad Soden, Germany) was preincubated with polyphenol for 3 min before the
reaction was started with the addition of potassium arachidonate. The num-
bers at the traces denote residual activities, in percentage of control values,
corrected for baseline drift. Modified from reference 34, with permission.

**SUPPRESSION OF MPO- AND PEROXYNITRITE-
MEDIATED MODIFICATIONS OF LDL**

Another oxidant enzyme with pronounced effects on inflam-
ination and atherogenesis is MPO (17, 40). Unlike lipoxygen-
ases, MPO is a hemoprotein dimer. MPO occurs in azurophilic
granules of neutrophils, macrophages, and other phagocytes and
is released into the extracellular space after activation of these
cells by inflammatory stimuli. This process apparently also oc-
curs in atherosclerotic lesions, because MPO-derived products
were found in human atherosclerotic tissue (41). The biological role of MPO was long regarded solely with respect to its reaction with hydrogen peroxide and chloride, forming hypochlorous acid, as part of the organism’s defense system against foreign microbes. However, the discovery that nitrite is also a MPO substrate (26, 42) opened a new era of contemporary knowledge regarding the biochemical features of MPO. A close connection to the metabolism of NO is obvious and was supported by the report that MPO also acts as a NO oxidase (43).

In vitro, MPO is capable of binding to LDL, catalyzing oxidative modification and rendering it atherogenic (44, 45). Therefore, study of the interaction of MPO with LDL with and without flavonoids is a promising approach to elucidate putative beneficial effects of dietary flavonoids in atherosclerosis.

MPO has been reported to cause lipid peroxidation of LDL in the presence of nitrite (27). We studied this reaction in more detail (46). Surprisingly, chloride was not able to support MPO-mediated lipid peroxidation of LDL, and its presence was not required for the nitrite-supported, lipid peroxidation of LDL. A moderate level of MPO-mediated lipid peroxidation, apparently accounted for by LDL-bound tyrosines, was strongly stimulated by low concentrations of nitrite. The effect of nitrite was significant at 5 μmol/L, and half-maximal stimulation of conjugated diene formation in LDL in the presence of chloride was achieved with 7 μmol/L nitrite (46). This concentration of nitrite is within the range found in arterial tissue (47), which is the putative site of oxidative modification of LDL during atherogenesis in vivo. Therefore, a biological role of the MPO/nitrite system in atherogenesis may be considered.

The MPO/nitrite-mediated lipid peroxidation of LDL was effectively blocked by the cocoa polyphenol (−)-epicatechin, the corresponding procyanidins, and other flavonoids (46, 48).

<table>
<thead>
<tr>
<th>Enzyme, species</th>
<th>Substrate used</th>
<th>Activity</th>
<th>Assay method</th>
<th>IC₅₀ (μmol/L)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Lipoxygenase, human</td>
<td>Arachidonic acid</td>
<td>Dioxygenase</td>
<td>5-H(p)ETE formation, HPLC</td>
<td>22</td>
<td>28</td>
</tr>
<tr>
<td>5-HpETE</td>
<td>Leukotriene A₄ synthase</td>
<td></td>
<td>5,12-Dihydroxy-HETE formation, HPLC</td>
<td>50</td>
<td>28</td>
</tr>
<tr>
<td>12-Lipoxygenase</td>
<td>Arachidonic acid</td>
<td>Dioxygenase</td>
<td>Oxygen uptake</td>
<td>15</td>
<td>34</td>
</tr>
<tr>
<td>Platelet-type, human</td>
<td>Arachidonic acid</td>
<td>Dioxygenase</td>
<td>12-H(p)ETE formation, HPLC</td>
<td>ND</td>
<td>34</td>
</tr>
<tr>
<td>Leukocyte-type, porcine</td>
<td>Arachidonic acid</td>
<td>Dioxygenase</td>
<td>Oxygen uptake, conjugated dienes</td>
<td>60</td>
<td>34</td>
</tr>
<tr>
<td>15-Lipoxygenase-1</td>
<td>Linoleic acid</td>
<td>Dioxygenase</td>
<td>Oxygen uptake</td>
<td>ND</td>
<td>34</td>
</tr>
</tbody>
</table>

TABLE 1

Inhibitory efficiency of (−)-epicatechin toward some lipoxygenases

1 HETE, hydroxyeicosatetraenoic acid; 5-HpETE, 5S-hydroxyperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid; 5-H(p)ETE, 5-HpETE measured after reduction as the most stable corresponding hydroxy derivative; 12-H(p)ETE, 12S-hydro(peroxy)-5Z,8Z,10E,14Z-eicosatetraenoic acid; 15-H(p)ETE, 15S-hydro(peroxy)-5Z,8Z,11Z,14Z-eicosatetraenoic acid; ND, not determined, but pronounced inhibitory effect was qualitatively observed.

FIGURE 3. Inhibition of human 5-lipoxygenase (dark shaded columns) and rabbit 15-lipoxygenase-1 (light shaded columns) activities by procyanidin fractions isolated from seeds of Theobroma cacao. 5-Lipoxygenase activity (formation of 5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid, analyzed as 5-HETE) was assayed as for Figure 1. The activity of rabbit reticulocyte 15-lipoxygenase (prepared as described in reference 37) was measured as for Figure 2, with the exception that the reaction was started with the addition of 20 μL of 5.3 mmol/L potassium linolate in 0.1 mol/L potassium phosphate (pH 7.4) containing 4% (wt:vol) sodium cholate, with a final volume of 400 μL. The final concentrations of cocoa procyanidins (isolated as described in reference 36) were 50 μEq/L and 100 μEq/L for the 5-lipoxygenase and 15-lipoxygenase assays, respectively. Values are mean ± SD (n = 3–5). NS, not significant, compared with the vehicle control (2% 2-methoxyethanol). Modified from reference 34, with permission, including data from reference 28.
inhibitory effects of flavonoids occurred through 2 different actions, ie, dose-dependent prolongation of the lag phase of conjugated diene formation at concentrations of ~1 μmol/L and lowering of the reaction rate of the propagation phase of lipid peroxidation at somewhat higher concentrations (~2 μmol/L). The mode of action of flavonoids with MPO was quite different from that with lipoxygenases. Flavonoids are not MPO inhibitors per se; they even serve as MPO substrates. For example, during the reaction of MPO with (−)-epicatechin in the presence or absence of LDL, a new chromophore with an absorption maximum at 435 nm was formed (48), the structure of which remains to be defined. It is tempting to speculate that MPO-mediated dehydrogenation of (−)-epicatechin or other flavonoids leads to several types of semiquinones, quinones, and quinone methides, which may be less functional as antioxidants than the parent flavonoids. The overlap of antioxidant actions of flavonoids on the MPO/nitrite reaction system with MPO-mediated conversion of flavonoids may explain the lag-phase phenomenon at low concentrations of flavonoids.

The MPO/nitrite system also caused protein tyrosine nitration in LDL; however, this reaction required higher concentrations of nitrite (≥ 100 μmol/L) than did formation of conjugated dienes in the lipid moiety of LDL. This reaction was suppressed by (−)-epicatechin at concentrations as low as 0.1 μmol/L (46).

The diagram in Figure 4 depicts the interactions of nitrite and flavonoids with the catalytic cycle of MPO, as well as the putative mode of protection of LDL by flavonoids against oxidative and nitrating modification. The involvement of NO2· radicals in the MPO/nitrite reaction system and the in vivo role of this system appear to be well substantiated by recent studies by Hazen and coworkers (49, 50).

Peroxynitrite is another deleterious product of NO. Bolus addition of this compound to LDL caused only minute lipid peroxidation but strong tyrosine nitration of the apoprotein, with the latter being independent of the presence of CO2 (46). Again, 0.1 μmol/L (−)-epicatechin strongly suppressed this reaction. In other systems, this major cocoa flavanol selectively attenuated nitrating reactions of peroxynitrite at low concentrations, whereas suppression of oxidation reactions by this compound required higher concentrations of (−)-epicatechin (51).

Some protective actions of (−)-epicatechin against several kinds of oxidative and nitrating modifications of human LDL we observed are compiled in Table 2. These data demonstrate that cocoa flavanols are universal protectors of human LDL against various types of proatherogenic modifications. This conclusion was corroborated by a recent report that intake of flavan-3-ol–rich cocoa products decreased significantly the oxidative susceptibility of LDL ex vivo, although the total antioxidative capacity of plasma was not changed under those conditions (52).

**TABLE 2**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Reaction/criterion</th>
<th>EC50 1 (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloperoxidase (nitrite)</td>
<td>Lipid peroxidation, prolongation of lag phase</td>
<td>~2</td>
</tr>
<tr>
<td></td>
<td>Lipid peroxidation, decrease of reaction rate</td>
<td>~5</td>
</tr>
<tr>
<td></td>
<td>Tyrosine nitrination</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Myeloperoxidase (tyrosine)</td>
<td>Lipid peroxidation, extent</td>
<td>~5</td>
</tr>
<tr>
<td>Cu2+</td>
<td>Lipid peroxidation, prolongation of lag phase</td>
<td>0.6</td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>Tyrosine nitrination</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

1 Effective concentration to achieve either 50% inhibition or doubling of lag phase.
healthy control subjects, and doubled FMD from 3.4 ± 0.05% to 6.3 ± 0.6% \((P < 0.001)\) after 2 h. Plasma concentrations of RNO were correlated with FMD \(r = 0.42, P = 0.02\), which suggests that the improvement of endothelial function might be attributable to increased bioavailability of NO. The changes occurred transiently 2 h after ingestion, as determined in a separate pilot study addressing the time dependence \(54\). In contrast, these changes did not occur among individuals who received either water or a low-flavanol cocoa drink, which excluded effects of beverage constituents other than flavanols. The changes in RNO and FMD caused by the high-flavanol cocoa beverage proved to be highly selective, because resting diameter and endothelium-independent dilation of the brachial artery \(12.9 \pm 1.8\%\), forearm blood flow at rest and reactive hyperemia, blood pressure, heart rate, and plasma nitrite and nitrate concentrations did not change under these conditions.

The maximal effects of the high-flavanol cocoa beverage coincided with the peak of \((\sim)-epicatechin metabolites in plasma \(57\), both occurring \(~2\) h after intake. This observation defines them as rapid actions. Therefore, they cannot be attributed to time-dependent increases in the expression of endothelial NO synthase, which was recently reported for red wine, another important source of dietary flavanols \(22, 58\). Rather, the observed actions may occur either at the level of cell signaling or at the level of endothelial NO synthase activity. Together with the data reported in the literature, these data indicate that the NO-promoting actions of dietary flavanols are attributable to more than one mechanism.

**FIGURE 5.** Effects of high-flavanol and low-flavanol cocoa drinks on RNO concentrations in plasma (A) and FMD (B) among individuals at risk for cardiovascular diseases. The test subjects (11 male and 9 female subjects) had at least one major cardiovascular risk factor (hypertension, hypercholesterolemia, diabetes mellitus, and/or smoking) \(55\). Exclusion criteria were elevated C-reactive protein concentrations \((>0.005\ \text{g/L})\), atrial fibrillation, acute coronary syndrome, or heart failure (New York Heart Association class III or IV). The individuals refrained from smoking and drinking caffeine-containing beverages and fasted for at least 12 h before the study. Analyses were performed 2 h after ingestion of 100 mL of either a high-flavanol cocoa drink (containing 176 mg of total flavanols) or a low-flavanol cocoa drink (containing <10 mg of flavanols), in a randomized, double-blind, crossover manner. Plasma RNO concentrations were measured after treatment of plasma with 0.1 volume of 5% sulfanilic acid in 1 mol/L HCl and subsequent reductive cleavage, with a reaction mixture containing 45 mmol/L iodide and 10 mmol/L triiodide, to NO, which was assayed on the basis of its chemiluminescent reaction with ozone \(24\). Endothelial function was assessed non-invasively by measuring FMD of the brachial artery with high-resolution ultrasonography, after 5 min of lower arm occlusion \(56\). Values are mean ± SEM \(n = 20\). *Significant differences from baseline values before ingestion of the drink \((P < 0.001, \text{paired } t\text{-test})\) and significant differences with the high-flavanol drink, compared with the low-flavanol control drink \((P = 0.005 \text{ in } A \text{ and } P < 0.001 \text{ in } B, \text{paired } t\text{-test})\). Statistical analyses were performed with SPSS for Windows, version 11.0.1 (SPSS Inc, Chicago, IL). Data from reference 54.

**DISCUSSION**

**General implications regarding flavonoid actions in vivo**

We provided evidence that flavan-3-ols and their procyanidins are general inhibitors of mammalian lipoxygenases at the enzyme level. The lipoxygenase-inhibiting potencies of \((\sim)-epicatechin and related flavonoids may therefore be considered to contribute to the antioxidative potential of these polyphenols. Whether this is actually the case for 5-lipoxygenase in vivo remains to be ascertained in future studies, which may include measurement of urinary excretion of leukotriene \(E_4\) and estimation of the 5-lipoxygenase-mediated formation of arachidonic acid products (mainly \(5\)-HETE and leukotriene \(B_4\)) from ionophore-stimulated neutrophils ex vivo. A finding supporting this assumption is that intake of procyanidin-rich chocolate among human subjects led to significant decreases in the plasma concentrations of cysteinyl leukotrienes, which are metabolites of the 5-lipoxygenase pathway of arachidonic acid metabolism, in parallel with increases in the plasma concentrations of \((\sim)-epicatechin metabolites \(11\)). Although the \(IC_{50}\) values for the various isolated lipoxygenases proved to be higher than the achievable plasma concentrations \(57, 59\) and most plasma \((\sim)-epicatechin is present as conjugates (eg, glucuronides), the 5-lipoxygenase inhibition appears to be of biological relevance, inasmuch as no information is available regarding the distribution of flavonoids and their metabolites in compartments of the organism other than blood plasma. We demonstrated that \((\sim)-epicatechin is accumulated in murine aortic endothelial cells and thus exerts intracellular protective actions \(60\). This observation suggests that the plasma concentrations of flavanol metabolites are different from the actual intracellular concentrations.

The pertinent literature shows the tendency to overrate the biological role of the plasma metabolites of \((\sim)-epicatechin and other flavonoids, which are thought by many authors to be both final metabolites and bioactive forms. Several reports are not in line with this suggestion. Flavonoid glucuronides were found to be deconjugated by various human cells and thus taken up as aglycones \(61\). In a study with \([4-\text{C}]\text{quercetin among human...}
subjects, the urinary recovery of radioactivity after oral ingestion of this isotope was as low as 3.3–5.7%, despite a high absorption rate of ~50%, whereas a large percentage was found as $^{14}$CO$_2$ in expired air. Furthermore, the half-life of the labeled flavonoid was rather long, ranging from 20 to 72 h (62). Together, these observations argue that flavonoid glucuronides may serve as transport forms in plasma, rather than as final metabolites.

**Biological consequences of the inhibition of lipoxygenase activities**

The biological role of 5-lipoxygenase is closely connected with the biosynthesis of leukotrienes, and the inhibition of human 5-lipoxygenase by cocoa flavonoids suggests antileukotriene actions of these compounds, which may confer some antiinflammatory, vasoprotective, and antibronchoconstrictory capacity. Leukotriene B$_4$ causes adherence of neutrophils to endothelial cells and is a potent chemotactic agent for these and other inflammatory cells. It also stimulates release of lysosomal enzymes and generation of superoxide anion in neutrophils. The cysteinyl leukotrienes increase vascular permeability and contract airway smooth muscle (8–10).

The subfamily of reticulocyte-type 12/15-lipoxygenases (including human and rabbit 15-lipoxygenase-1) differ from other mammalian lipoxygenases. This refers to the capability of directly dioxygenating polyenoic fatty acids in esterified form, such as those present in membrane phospholipids and in cholesterol esters of plasma lipoproteins, without requiring a lipid-cleaving enzyme. Unlike 5-lipoxygenase and platelet-type 12-lipoxygenase, the reticulocyte-type lipoxygenases are thus catalysts of enzymatic lipid peroxidation (for details, see reference 33 and references cited therein). In addition to involvement in the biologically programmed maturational breakdown of mitochondria in reticulocytes (63), a role in oxidative modification of LDL has been discussed (13, 14). Oxidative modification of LDL by 15-lipoxygenase-1, MPO, peroxynitrite, thiols, or transition metals renders it atherogenic (16). According to the oxidative hypothesis of atherosclerosis (16), this process is thought to play a fundamental role in early stages of the development of atherosclerotic lesions. If so, then any agent that interferes with oxidative modification of LDL should be protective for vascular endothelium, including that of coronary arteries. Most of the agents proposed to be involved in oxidative modification of LDL have been found by us to be counteracted by dietary polyphenols, in particular by (−)-epicatechin.

Detailed studies of the 15-lipoxygenase-1–mediated oxidation of LDL (64) revealed that this process involves 2 steps, ie, direct enzymatic attack on cholesteryl linoleate in LDL, leading to predominately specific oxygenation product, and lipoxygenase-mediated nonenzymatic lipid peroxidation, leading to nonspecific secondary reaction products. The longer the reaction continues, the greater is the share of nonspecific lipid peroxidation products, which can eventually predominate, although this process is enzymatically initiated. Dietary flavonoids appear to suppress both steps of this process; they inhibit 15-lipoxygenase-1 activity (34, 35) and they are known to effectively scavenge lipid-derived free radicals, which are intermittently formed during lipoxygenase-mediated, nonenzymatic, lipid peroxidation. Because the flavonoids may also scavenge secondary free radicals during oxidative modification of LDL evoked by endogenous oxidants other than 15-lipoxygenase-1, their antioxidative protection of LDL and, consequently, of the cardiovascular system appears to be universal. On the basis of these data taken together, we propose that inhibition of 15-lipoxygenase-1 activity and of the complex reactions with LDL contributes to the effects of cocoa flavonoids on the vascular endothelium.

The biological role of platelet 12-lipoxygenase is not well understood (65). This enzyme appears to be not involved in platelet aggregation and adhesion; therefore, the suppression of these phenomena by cocoa and wine flavonoids (66–68) cannot be attributed to inhibition of 12-lipoxygenase but rather is attributable to enhanced bioactivity of NO (20–22). Two types of biologically active eicosanoids are formed via the 12-lipoxygenase pathway of arachidonic acid metabolism, ie, 12S-hydro(pero)xy-5Z,8Z,10E,14Z-eicosatetraenoic acid and heposxilin A$_3$ and B$_3$ (69). In most cells possessing platelet-type 12-lipoxygenase, the formation of 12-HETE prevails. Inhibition of glutathione peroxidases causes a shift toward heposxilin synthesis (70). For 12-HETE, a role in several processes related to tumor metastasis has been proposed (71), but other biological actions have also been described (72). The main biological actions of heposxilin A$_3$ are related to its ability to release Ca$^{2+}$ from intracellular stores in human neutrophils and other cells (69). Whether intervention in the 12-lipoxygenase pathway contributes to the potential beneficial effects of dietary flavonoids remains to be clarified.

**Lipoxygenases and free radicals**

It should be stressed that the inhibition of lipoxygenases by flavonoids and other polyphenols is not necessarily a consequence of the free radical-scavenging properties of the polyphenols. Although intermediate free radicals are formed during the catalytic cycles of lipoxygenase-catalyzed reactions (33), these radicals remain tightly bound at the active site of the enzyme and are not accessible to such well-known lipophilic radical scavengers as 2,6-di-tert-butyl-4-methylphenol and probucol (73), as well as α-tocopherol. However, a variety of polyphenols are concomitantly free radical scavengers and lipoxygenase inhibitors. The most well-characterized example is nordihydroguaiaretic acid. For soybean lipoxygenase L-1, the inhibitory effect of nordihydroguaiaretic acid is obviously attributable to reduction of the active ferric form of the enzyme to the silent ferrous form, with interruption of the catalytic cycle of the dioxygenase reaction (74).

Intermediate enzyme-bound free radicals formed during lipoxygenase catalysis can be released from the active site under certain circumstances, to initiate secondary lipid peroxidation or other deleterious consecutive reactions. The extent of these side processes depends on the reaction conditions, particularly the type of substrate for the lipoxygenase. Lipophilic free radical scavengers selectively suppress such side reactions, although they are not true lipoxygenase inhibitors (73, 75). Dietary flavonoids and other polyphenols obviously combine the 2 properties, however, and suppress complex lipoxygenase-catalyzed processes in a dual manner.

**Possible role of quercetin in the dietary value of cocoa products**

Although (−)-epicatechin and related procyanidins contribute most of the total polyphenol content of cocoa products, the possible role of the quercetin content should not be overlooked. The
total amount of quercetin and related flavonols in cocoa has been reported to be 0.03% by wt (76), which corresponds to approximately one-tenth of the content of flavan-3-ols. We observed 37-fold and 15-fold higher inhibitory potencies of quercetin, compared with (−)-epicatechin, toward 5-lipoxygenase and 15-lipoxygenase-1, respectively; therefore, the lipoxygenase-inhibiting activity of the total mixture of cocoa flavonoids may be partly attributable to quercetin.

Cocoa polyphenols and NO metabolism

The data from our human study (54) indicated that the bioactivity of NO is markedly improved after ingestion of cocoa polyphenols and that the vascular endothelium is a major target of dietary polyphenols. These data are in line with those from other studies of human subjects who received other flavan-3-ol-containing beverages (18, 19) or of rats (21), as well as with studies with endothelial cells (22). The NO-promoting effects of cocoa polyphenols may also contribute to the recently reported decreases in blood pressure among elderly individuals after intake of dark chocolate (77).

In vitro investigations at the cellular and subcellular levels are needed to define how the NO-promoting action of cocoa polyphenols is brought about. The bioactivity of NO is expected to be the result of both synthesis of NO and its conversion to bioactive storage forms and other metabolites, including those exerting deleterious actions. It is tempting to speculate that dietary polyphenols interfere with NO metabolism at several levels. They may favor the synthesis of NO and concomitantly suppress effects of deleterious metabolites such as superoxide, peroxynitrite, and nitrogen dioxide. Another possibility could involve inhibition of the oxidation of NO to nitrite, a process that is not well understood. Suppression of the modifications of LDL by MPO/nitrite or peroxynitrite, as we observed, might also contribute to the complex effects of dietary polyphenols on NO metabolism.

CONCLUSIONS

Inhibitory effects on mammalian lipoxygenases, particularly 5-lipoxygenase and 15-lipoxygenase-1, may contribute to the potentially beneficial actions of cocoa flavonoids, although clear in vivo evidence is still lacking. Another target of these polyphenols is MPO, with the NO metabolite nitrite as substrate. Finally, our human study and other studies with human subjects and laboratory animals suggest that improvement of NO bioactivity is a pivotal action of dietary flavonoids.

We thank H Kühn (Berlin) and VA Kostyuk (Minsk, Belarus) for fruitful cooperation, as well as graduate students C Sadik, T Kraemer, and I Prakosay for participation in parts of this work. We gratefully acknowledge C Kwik-Uribe and M Kelm (Mars Inc, Hackettstown, NJ) for flavonoid analyses.

REFERENCES

Reversing the deleterious effects of aging on neuronal communication and behavior: beneficial properties of fruit polyphenolic compounds

James A Joseph, Barbara Shukitt-Hale, and Gemma Casadesus

ABSTRACT

Despite elegant research involving molecular biology studies and determination of the genetic mechanisms of aging, practical information on how to forestall or reverse the deleterious effects of aging may be years away. If this is the case, then it is prudent to try to establish other methods that can be used now to alter the course of aging. Numerous epidemiologic studies have indicated that individuals who consume diets containing large amounts of fruits and vegetables may reduce their risk for developing age-related diseases such as Alzheimer disease. Research from our laboratory suggested that dietary supplementation with fruit or vegetable extracts high in antioxidants (e.g., blueberry or spinach extracts) might decrease the enhanced vulnerability to oxidative stress that occurs in aging. These reductions might be expressed as improvements in motor and cognitive behavior. Additional research suggested that mechanisms in addition to antioxidant and antiinflammatory activities might be involved in the beneficial effects of these extracts; the most important of these might be their ability to increase cellular signaling and neuronal communication. Am J Clin Nutr 2005;81(suppl):313S–6S.

KEY WORDS  Aging, oxidative stress, inflammation, polyphenolic compounds, brain, signaling

INTRODUCTION

It is well known that many age-related behavioral changes in motor and cognitive performance occur even in the absence of specific, age-related, neurodegenerative diseases such as Alzheimer disease or Parkinson disease. Research discussed in this review suggests that the aged brain may provide a sensitive environment for the development of these diseases, leading to even more severe deficits in memory and/or motor function. This could result in increases in the number of elderly patients in need of hospitalization and/or custodial care. Therefore, unless some means is found to reduce these age-related decrements in neuronal function, health care costs will continue to increase exponentially. In both financial and human terms, it is extremely important to explore methods to retard or reverse age-related neuronal deficits and their subsequent behavioral manifestations. In this review, we describe the motor and cognitive deficits in behavior, show how these deficits are related to increased vulnerability to oxidative stress and inflammation, and describe the possible role of nutritional supplementation with fruits containing large amounts of polyphenols, such as anthocyanins, in reversing or forestalling these deficits.

BEHAVIORAL DECREMENTS

A great deal of research indicates the occurrence of numerous neuronal and behavioral deficits during normal aging. These changes may include decrements in calcium homeostasis (1) and in the sensitivity of several receptor systems, most notably the dopaminergic (2, 3), muscarinic (4, 5), opioid (6), and adrenergic (7) receptor systems. These losses in neuronal function may be expressed ultimately as alterations in both cognitive (8) and motor (9) behaviors. The memory deficits are noted in cognitive tasks that require the use of spatial learning and memory (10), whereas motor function deficits may include decreases in balance, muscle strength, and coordination (9). These changes have been demonstrated in many studies and appear to occur among both animals (10) and human subjects (11). Alterations in memory appear to occur primarily in secondary memory systems and are reflected in the storage of newly acquired information (12). Deficits in motor performance are thought to be the result of alterations in the striatal dopamine or cerebellar systems, which show marked neurodegenerative changes with age (12, 13).

Research shows that the hippocampus mediates place learning, whereas the prefrontal cortex is critical for acquiring the rules that govern performance of particular tasks (i.e., procedural knowledge). It appears that the dorsomedial striatum regulates spatial orientation that involves response and cue learning (14). Importantly for this review, substantial research indicates that factors such as oxidative stress (15) and inflammation (16, 17) may be major contributors to the behavioral decrements seen in aging.

In animal models, cognitive function is usually measured in a maze and motor function is measured with a battery of different tasks that require the use of spatial learning and memory (10), whereas motor function deficits may include decreases in balance, muscle strength, and coordination (9). These changes have been demonstrated in many studies and appear to occur among both animals (10) and human subjects (11). Alterations in memory appear to occur primarily in secondary memory systems and are reflected in the storage of newly acquired information (12). Deficits in motor performance are thought to be the result of alterations in the striatal dopamine or cerebellar systems, which show marked neurodegenerative changes with age (12, 13).

1 From the US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University, Boston (JAJ, BS-H), and the Institute of Pathology, Case Western Reserve University, Cleveland (GC).
2 Supported by USDA intramural grants, the Wild Blueberry Association of North America, and the US Highbush Council.
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tests, such as those that assess the time a rodent can remain on an accelerated (a slowly rotating rod). Maze procedures are used to assess learning (acquisition), working (short-term), and reference (long-term) memory functions. Reference memory is consistent among trials and is required for learning the general rules of any task (eg, swim to a platform) (18). In contrast, working memory describes the ability of the subject to hold specific information (places previously visited) in memory (18). Old rats were shown to have decrements in both reference and working memory in the Morris water maze (for review, see reference 15), the radial arm maze (for review, see reference 19), and the radial arm water maze (20).

OXIDATIVE STRESS AND INFLAMMATION IN AGING

Oxidative Stress

An abundance of data suggest that one of the most important factors mediating the deleterious effects of aging on behavior and neuronal function is oxidative stress (for review, see reference 21). The central nervous system appears to be especially vulnerable to the effects of oxidative stress, partially as a result of additional factors such as increases in the ratio of oxidized glutathione to total glutathione (22), significant lipofuscin accumulation (23) with bel-2 increases (24), increases in membrane lipid peroxidation (25), reductions in glutamine synthetase (26), reductions in redox-active iron (23, 27), and alterations in membrane lipids (28). Importantly, in addition to these considerations, it has been shown that, not only is the central nervous system particularly vulnerable to oxidative stress, but this vulnerability increases during aging (for review, see references 29 and 30). Research has also shown that, in addition to the factors discussed above (eg, reductions in glutathione concentrations) (22), oxidative stress vulnerability in aging may be the result of 3 other factors, namely, alterations in the membrane microenvironment, alterations in calcium-buffering ability, and differential vulnerability of neurotransmitter receptors. Findings suggest that age-related changes in the neuronal plasma membrane molecular structure and physical properties (eg, increased rigidity) may play a role in increasing vulnerability to oxidative stress and inflammation (29, 31).

Inflammation

Evidence also suggests that inflammatory events in the central nervous system may play an important role in aging. By middle age, there is increased glial fibrillary acidic protein expression (32); in old age, expression occurs even in the absence of an inflammatory stimulus (33). In conjunction with this observation, it was reported that tumor necrosis factor-α is produced in greater amounts during cytotoxic reactions among elderly subjects (34) and that neuronal inhibition of glial activities may be lost during aging (35). Other studies reported increases in tumor necrosis factor-α and interleukin-6 concentrations in the sera of aged mice (36) and human subjects (37). In fact, it has been suggested that up-regulation of C-reactive protein may represent one factor in biological aging (38).

Another important point is that there may be important interactions of reactive oxygen species-generating agents and cytokines. For example, Manev and Uz (39) demonstrated increased sensitivity of senescent rats to central injections of kainic acid, an excitotoxin that induces inflammatory reactions involving factors such as cytokines, complement proteins, and adhesion molecules. These may represent extracellular signals that act in concert with reactive oxygen species to initiate neuronal functional deficits and glial cell-neuron interactions (40–42). Therefore, it appears that the increases in sensitivity with respect to oxidative stress and inflammation that are observed in senescence may be involved in mediating age-related deficits. Similarly, it appears that treatments (eg, heavy-particle irradiation) that increase oxidative and/or inflammatory stressors may produce behavioral deficits that parallel those seen in aging (43–45). In addition, research has shown that the induction of neuronal/glial inflammation through central administration of lipopolysaccharide, a bacterial toxin that is a potent inflammatory agent, can reproduce many of the behavioral, inflammatory, neurochemical, and neuropathologic changes seen in the brains of patients with Alzheimer disease in some similar regions (eg, cingulate cortex), as well as producing changes in spatial learning and memory behavior (16, 17, 46, 47).

Previous studies (16, 17, 46–48) showed that chronic (28–37-d) infusion of lipopolysaccharide into the ventricles of young rats increased several markers of inflammation. These changes included but were not limited to increased numbers of activated astrocytes, increased numbers and densities of activated microglia (particularly within the hippocampus, cingulate cortex, and basal forebrain), increased concentrations of cytokines, degeneration of hippocampal pyramidal neurons, and impairment of working memory (16, 17, 46, 48).

EFFECTS OF FRUIT AND VEGETABLE SUPPLEMENTATION ON BEHAVIORAL AND NEURONAL DEFICITS IN AGING

Antioxidants have been studied for their effectiveness in reducing the deleterious effects of brain aging and behavior in many studies (49–51). Although many of those experiments yielded mixed results, research from our laboratory suggested that the combinations of antioxidant/antiinflammatory polyphenolic compounds found in fruits and vegetables may show efficacy in aging. Plants, including food plants (fruits and vegetables), synthesize a vast array of secondary chemical compounds that, although not involved in primary metabolism, are important for a variety of ecologic functions that enhance the plant’s ability to survive. Interestingly, these compounds may be responsible for the multitude of beneficial effects that have been reported for fruits and vegetables, with an array of health-related bioactivities. Many studies (52–56) have suggested that the most important benefits of such compounds may be derived from their antioxidant and antiinflammatory properties. Until very recently, however, most dietary agents used to alter behavioral and neuronal effects with aging included nutritional supplements such as vitamins C and E, garlic (49), herbal supplements (eg, ginseng, Ginkgo biloba, and ding lang) (50), and dietary fatty acids (for review, see reference 51).

We thought that, given the considerable antioxidant/antiinflammatory potential of fruits and vegetables, they might show some efficacy in reducing the deleterious effects of aging on neuronal function and behavior. In our first study, we used fruits and vegetables identified as being high in antioxidant activity in the oxygen radical absorbance capacity assay (52–54) and showed that long-term (from 6 to 15 mo of age) feeding of F344
rats with an AIN-93 diet supplemented with strawberry or spinach extract (1–2% of the diet) or vitamin E (500 IU) retarded age-related decrements in cognitive and neuronal function compared to an AIN-93 diet alone. Results indicated that the supplemented diets could prevent the onset of age-related deficits in several indices (eg, cognitive behavior and Morris water maze performance) (55).

In a subsequent experiment (56), we found that dietary supplementation (for 8 wk) with spinach, strawberry, or blueberry extracts in an AIN-93 diet was effective in reversing cognitive deficits in Morris water maze performance function among aged (19-mo-old) F344 rats. However, only the blueberry supplement-treated group exhibited improved performance on tests of motor function. Specifically, the blueberry supplement–treated group displayed improved performance on rod walking and latency to falling from an accelerating rotarod. Both of these tests rely on balance and coordination. None of the other supplement-treated groups differed from the control group in these tasks.

Although examinations of reactive oxygen species production in brain tissue obtained from animals in the various diet groups indicated that the striata obtained from all of the supplement–treated groups exhibited significantly lower reactive oxygen species concentrations (assayed as 2,7'-dichlorofluorescein diacetate) than did the control group, these decreases did not appear to be sufficient to account for the observed significant beneficial effects of blueberry supplementation on motor and cognitive function. It was clear from this study (56) and a subsequent study (57) that the significant effects of blueberries on both motor and cognitive behavior might involve actions other than antioxidant or antiinflammatory activities. Research from several sources suggests that at least some of these actions may include alterations in signaling. It is known that flavonoids can have potent effects on cell signaling. For example, delphinidin inhibits endothelial cell proliferation and cell cycle progression through extracellular signal-regulated kinase (ERK)-1/2 activation (58), whereas grape seed proanthocyanidin can reduce ischemia/reperfusion-induced activation of JNK-1 and c-Jun and reduce cardiomycyte apoptosis (59). Additional research indicates that phytochemicals can selectively regulate multiple signaling pathways at the level of transcription, especially those involving mitogen-activated protein kinase (60). In this regard, in the study by Joseph et al (56), significant increases in several indices of neuronal signaling (eg, muscarinic receptor sensitivity), as well as reversals in age-related dysregulation in 45Ca2+ buffering capacity, an important index of neuronal dysfunction in aging (1), were observed in the diet groups but not in the control group.

Additional evidence for these signaling changes was seen in a recent study (61) in which APP/PS1 transgenic mice, which have genetic mutations that promote the production of β-amyloid and then hallmark Alzheimer disease-like plaques in several brain regions, were given blueberry supplements (as in reference 56). The supplementation was begun at 4 mo of age and continued until the mice reached 12 mo of age. The mice were then tested for their performance in a Y-maze. The results indicated that the blueberry supplement–treated mice showed performance similar to that of non–supplement–treated transgenic animals. Examination of the brains of the mice revealed no differences between the supplement–treated and non–supplement–treated APP/PS1 mice in the number of plaques. It appeared that there was a discrepancy between plaque deposition and Y-maze performance, because performance did not decline among the blueberry supplement–treated APP/PS1 mice but did show decrements among the APP/PS1 mice maintained on the control diet.

Additional analyses revealed that the blueberry supplement–treated APP/PS1 mice showed higher concentrations of hippocampal ERK, as well as striatal and hippocampal protein kinase C, than did the APP/PS1 mice maintained on a control diet. Both protein kinase C and ERK have been shown to be important in the conversion of short-term memory to long-term memory (62). These findings suggested that blueberry supplementation might prevent cognitive deficits by directly enhancing neuronal signaling and offsetting any putative deleterious effects of amyloid deposition. The data also revealed that blueberry supplementation, in addition to enhancing mitogen-activated protein kinase signaling, increased the sensitivity of muscarinic receptors (increasing striatal, carbachol-stimulated, GTPase activity). As mentioned above, these receptors have been implicated in cognitive function for many years (8).

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REFERENCES

316S JOSEPH ET AL


Polyphenols and disease risk in epidemiologic studies
deepest- to 4
Ilja CW Arts and Peter CH Hollman

ABSTRACT
Plant polyphenols, a large group of natural antioxidants, are serious candidates in explanations of the protective effects of vegetables and fruits against cancer and cardiovascular diseases. Epidemiologic studies are useful for evaluation of the human health effects of long-term exposure to physiologic concentrations of polyphenols, but reliable data on polyphenol contents of foods are still scarce. The aim of this review is to summarize available epidemiologic data on the health effects of polyphenols, focusing on the flavonoid subclasses of flavonols, flavones, and catechins and on lignans. Data obtained to date suggest beneficial effects of both flavonoids and lignans on cardiovascular diseases but not on cancer, with the possible exception of lung cancer. There is a need for more research on stroke and lung diseases such as asthma and chronic obstructive pulmonary disease. Most studies to date have included only flavonols and flavones. With data becoming available for other polyphenols, these compounds should be included in future studies. Careful design of prospective studies is important to offset some of the major drawbacks of epidemiologic studies, including residual confounding (by smoking and other dietary factors) and exposure assessment. Am J Clin Nutr 2005;81(suppl):317S–25S.

KEY WORDS Review, epidemiology, polyphenols, flavonoids, flavonols, catechins, lignans, antioxidants, phytoestrogens, cancer, cardiovascular diseases, stroke

INTRODUCTION
Epidemiologic studies suggest a protective effect of vegetables and fruits against cancer and cardiovascular diseases (CVDs) (1, 2). Various hypotheses have been suggested to explain these beneficial effects of increased consumption of vegetables and fruits. An attractive hypothesis is that vegetables and fruits contain compounds that have protective effects, independent of those of known nutrients and micronutrients. Plant polyphenols, a large group of natural antioxidants ubiquitous in a diet high in vegetables and fruits, certainly are serious candidates. All plant phenols are derived from the common intermediate phenylalanine, or its close precursor shikimic acid, through the shikimic acid pathway in plants. They can be divided into at least 10 different classes on the basis of their general chemical structures, with the common characteristic being at least one aromatic ring structure with one or more hydroxyl groups. A large variety of plant (poly)phenols exist, including cinnamic acids, benzoic acids, flavonoids including proanthocyanidins, stilbenes, coumarins, lignans, and lignins. Within each family of plant phenols, many compounds may be present. For example, >6000 different flavonoids occurring in plants have been described.

In addition to their antioxidant properties, polyphenols show several interesting effects in animal models and in vitro systems; they trap and scavenge free radicals, regulate nitric oxide, decrease leukocyte immobilization, induce apoptosis, inhibit cell proliferation and angiogenesis, and exhibit phytoestrogenic activity (3–6). These effects may contribute to their potentially protective role in cancer and CVDs. The question remains of whether these data are relevant for human disease outcomes, where exposure to polyphenols is chronic and at relatively low concentrations, depending on bioavailability and metabolism. An important phenomenon is that, after absorption, polyphenols are subject to phase II metabolism, yielding methoxylated, glucuronidated, and sulfated compounds (7). This may greatly influence their bioactivity, but only a few studies have examined this to date. In addition, bacteria present in the human colon metabolize polyphenols. The major polyphenol metabolites are a variety of phenolic acids such as homovanillic acid (8). As a consequence, body tissues are exposed to high concentrations of these phenolic acids.

Although no information on causality can be obtained, epidemiologic studies are useful for evaluation of the human health effects of long-term exposure to physiologic concentrations of polyphenols. Reliable data on polyphenol contents of foods are needed for studies of the potential role of dietary polyphenols in cancer and CVD prevention. Comprehensive data are available only for the flavonoid subclasses of flavonols, flavones, and catechins, but data on other polyphenols, such as lignans, are forthcoming. In this article, we provide an overview of epidemiologic studies on the health effects of flavonols, flavones, catechins, and lignans conducted to date.

FLAVONOIDS

Studies
Of the 6 major classes of flavonoids, comprehensive data on their contents in foods are available only for the flavonols (quercetin,
kaempferol, and myricetin), flavones (apigenin and luteolin), and catechins ([+]–catechin, [+]-gallocatechin, [−]-epicatechin, [−]-epigallocatechin gallate, [−]-epigallocatechin gallate). The flavonoid data used in most epidemiologic studies were based on analyses conducted in the Netherlands (9–12) but were supplemented in some studies with data for additional food items. Recently, data became available for flavonones in Finnish foods (hesperetin, naringenin, and eriodictyol) (13). Except for one study from the United Kingdom (14), all epidemiologic studies of flavonoids are from the Netherlands, Finland, or the United States.

CVDs

To date, 12 cohort studies on flavonoid intake and the risk of coronary artery disease (CAD) and 5 cohort studies on the risk of stroke have been published (Table 1). Seven of these prospective studies found protective effects of flavonols and flavones or of catechins with respect to fatal or nonfatal CAD, and reductions of mortality risk were up to 65%. These studies were as follows: the Zutphen Elderly Study, with a small cohort of 805 men in the Netherlands after 5 and 10 y of follow-up monitoring (21, 24), the Finnish Mobile Clinic Health Examination Survey (significant among men only) (22), the Iowa Women’s Health Study, a cohort study of 34,500 women in the United States (20), the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study among 25,000 male smokers (19), the Dutch Zutphen Elderly Study (18), and the Rotterdam Study in the Netherlands, a cohort study of 4800 men and women (16). In a large cohort study of 35,000 male US health professionals, a suggestion of a reduction in coronary mortality rates with high flavonol intake was found only among men with a previous history of CAD [relative risk (RR): 0.63; 95% CI: 0.33, 1.20] (23). In contrast, a trend for increased CAD mortality rates (P for trend = 0.12) was found in the Caerphilly Study, a cohort study of 1900 Welsh men (14). It was suggested that the English habit of adding milk to tea (the Caerphilly Study, a cohort study of 1900 Welsh men (14)) could inhibit the absorption of flavonols, thus explaining the lack of protection of tea flavonoids against CAD. Proteins bind phenols efficiently and therefore might inhibit absorption from the gastrointestinal tract when consumed together with flavonoids. However, it was shown that the absorption of flavonols was not impaired with the addition of milk (27). Residual confounding by lifestyle factors

### Table 1

<table>
<thead>
<tr>
<th>Reference</th>
<th>Country</th>
<th>Population</th>
<th>Flavonoid</th>
<th>Comparison (high vs low intake)</th>
<th>Follow-up time</th>
<th>Outcome</th>
<th>No. of cases</th>
<th>Adjusted RR (high vs low)</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sesso et al, 2003 (15)</td>
<td>US</td>
<td>38 484 M</td>
<td>Flavonols, flavones</td>
<td>47.4 vs 8.9</td>
<td>6.9</td>
<td>Total CVD</td>
<td>519</td>
<td>0.80 (0.59, 1.09)</td>
<td>0.80</td>
</tr>
<tr>
<td>Geleijse et al, 2002 (16)</td>
<td>Netherlands</td>
<td>4807 MF</td>
<td>Flavonols</td>
<td>40.0 vs 16.8</td>
<td>5.6</td>
<td>Nonfatal MI</td>
<td>116</td>
<td>0.93 (0.57, 1.52)</td>
<td>—</td>
</tr>
<tr>
<td>Knekt et al, 2002 (13)</td>
<td>Finland</td>
<td>9131 MF</td>
<td>Flavonols, flavones, flavanones</td>
<td>M &gt; 26.9 vs &lt; 4.3; F &gt; 39.5 vs &lt; 8.5</td>
<td>28</td>
<td>CAD</td>
<td>681</td>
<td>0.93 (0.74, 1.17)</td>
<td>0.30</td>
</tr>
<tr>
<td>Arts et al, 2001 (17)</td>
<td>US</td>
<td>32 857 F</td>
<td>Catechins</td>
<td>74.8 vs 3.6</td>
<td>13</td>
<td>CAD</td>
<td>767</td>
<td>0.85 (0.67, 1.07)</td>
<td>—</td>
</tr>
<tr>
<td>Arts et al, 2001 (18)</td>
<td>Netherlands</td>
<td>806 M</td>
<td>Catechins</td>
<td>124.0 vs 25.3</td>
<td>10</td>
<td>CAD</td>
<td>90</td>
<td>0.49 (0.27, 0.88)</td>
<td>0.02</td>
</tr>
<tr>
<td>Hirvonen et al, 2001 (19)</td>
<td>Finland</td>
<td>25 372 M</td>
<td>Flavonols, flavones</td>
<td>17.8 vs 3.9</td>
<td>6.1</td>
<td>Nonfatal MI</td>
<td>1122</td>
<td>0.77 (0.64, 0.93)</td>
<td>—</td>
</tr>
<tr>
<td>Yochum et al, 1999 (20)</td>
<td>US</td>
<td>34 492 F</td>
<td>Flavonols, flavones</td>
<td>28.6 vs 4.0</td>
<td>10</td>
<td>CAD</td>
<td>438</td>
<td>0.62 (0.44, 0.87)</td>
<td>0.11</td>
</tr>
<tr>
<td>Hertog et al, 1997 (21)</td>
<td>Netherlands</td>
<td>804 M</td>
<td>Flavonols, flavones</td>
<td>41.6 vs 12.0</td>
<td>10</td>
<td>CAD</td>
<td>90</td>
<td>0.47 (0.27, 0.82)</td>
<td>0.01</td>
</tr>
<tr>
<td>Hertog et al, 1997 (14)</td>
<td>UK</td>
<td>1900 M</td>
<td>Flavonols, flavones</td>
<td>42.8 vs 13.5</td>
<td>14</td>
<td>CAD</td>
<td>131</td>
<td>1.60 (0.90, 2.90)</td>
<td>0.12</td>
</tr>
<tr>
<td>Knekt et al, 1996 (22)</td>
<td>Finland</td>
<td>2745 M</td>
<td>Flavonols, flavones</td>
<td>&gt;4.8 vs &lt;2.1</td>
<td>26</td>
<td>CAD</td>
<td>324</td>
<td>0.67 (0.44, 1.00)</td>
<td>0.12</td>
</tr>
<tr>
<td>Rimm et al, 1996 (23)</td>
<td>US</td>
<td>34 789 F</td>
<td>Catechins</td>
<td>40.0 vs 7.1</td>
<td>6</td>
<td>Nonfatal MI</td>
<td>486</td>
<td>1.08 (0.81, 1.43)</td>
<td>—</td>
</tr>
<tr>
<td>Arts et al, 2001 (18)</td>
<td>Netherlands</td>
<td>806 M</td>
<td>Catechins</td>
<td>124.0 vs 25.3</td>
<td>10</td>
<td>CAD</td>
<td>90</td>
<td>0.49 (0.27, 0.88)</td>
<td>0.02</td>
</tr>
<tr>
<td>Hirvonen et al, 2000 (25)</td>
<td>Finland</td>
<td>26 497 M</td>
<td>Flavonols, flavones</td>
<td>16.4 vs 4.2</td>
<td>6.1</td>
<td>Incident stroke</td>
<td>736</td>
<td>0.98 (0.80, 1.21)</td>
<td>0.81</td>
</tr>
<tr>
<td>Yochum et al, 1999 (20)</td>
<td>US</td>
<td>34 492 F</td>
<td>Flavonols, flavones</td>
<td>28.6 vs 4.0</td>
<td>10</td>
<td>Stroke</td>
<td>131</td>
<td>1.18 (0.70, 2.00)</td>
<td>0.83</td>
</tr>
<tr>
<td>Keli et al, 1996 (26)</td>
<td>Netherlands</td>
<td>352 M</td>
<td>Flavonols, flavones</td>
<td>33.3 vs 14.2</td>
<td>15</td>
<td>Incident stroke</td>
<td>42</td>
<td>0.27 (0.11, 0.70)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

1. MI, myocardial infarction; —, no data provided.
3. Mean, median, or category cutoff value.
4. Death, unless indicated otherwise.
5. 95% CI in parentheses.
6. Quartiles were constructed for men and women separately, but RR is provided for sexes combined only.
might have affected evaluation of the results of this study among Welsh men.

Two of 5 studies of flavonoid intake and stroke risk found an inverse association, ie, the Zutphen Elderly Study and the Finnish Mobile Clinic Health Examination Survey (Table 1). In the Zutphen Elderly Study, a protective effect was observed for flavonols and flavones (26) but not for catechins (18).

**Cancer**

Associations between the intake of flavonoids and the incidences of a variety of cancers have been studied in 7 prospective cohort studies (Table 2) and 4 case-control studies. Significant associations were observed only for lung cancer and colorectal cancer. Two Finnish studies with relatively low intakes of flavonols and flavones, ie, the Finnish Mobile Clinic Health Examination Survey (30) and the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (32), found inverse associations with lung cancer risk (RR: 0.53; 95% CI: 0.29, 0.97; and RR: 0.56; 95% CI: 0.45, 0.69, respectively). In contrast, a borderline positive association was found for colorectal cancer risk in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (RR: 1.70; 95% CI: 1.00, 2.70; P for trend = 0.10). For catechins, an inverse association was reported for rectal cancer (RR: 0.55; 95% CI: 0.32, 0.95; P for trend = 0.02), but not for colon cancer, among postmenopausal women in the United States. No evidence for an effect of flavonoid intake was found for the incidence of any epithelial cancer or cancer of the stomach, urinary tract, prostate, or breast. None of the case-control studies of prostate (34), lung (35), testicular (36), and ovarian (37) cancer found significant associations.

**Other chronic diseases**

Because of their antioxidant and antiinflammatory properties, flavonoids may also beneficially influence other chronic diseases involving oxidative stress or inflammation, such as rheumatoid arthritis and chronic obstructive pulmonary disease (COPD). Knekt et al (13) studied the associations between the intake of flavonols, flavones, and flavanones and the incidences of rheumatoid arthritis, type 2 diabetes mellitus, cataracts, and asthma among a cohort of 10 000 male and female participants in the Finnish Mobile Clinic Health Examination Survey (Table 2). A significant inverse association was observed only for asthma (RR: 0.65; 95% CI: 0.47, 0.90; P for trend = 0.04). This finding supported an earlier cross-sectional study, in which intake of flavonoids was beneficially associated with pulmonary function and symptoms of COPD. Pulmonary function (measured as forced expiratory volume in 1 s) was better among subjects in the highest quintile, compared with the lowest quintile, of intake of flavonols, flavones, and catechins (44 mL; 95% CI: 18, 69 mL). Catechin intake alone was most strongly associated with the forced expiratory volume in 1 s (130 mL; 95% CI: 101, 159 mL) and with all 3 symptoms of COPD [cough odds ratio (OR): 0.72; 95% CI: 0.58, 0.90; phlegm OR: 0.60; 95% CI: 0.47, 0.75; breathlessness OR: 0.69; 95% CI: 0.52, 0.90] (38).

**LIGNANS**

**Studies**

Plant lignans can be converted by human intestinal bacteria into the so-called enterolignans, ie, enterolactone and enterodiol. Enterolignans are found in biological fluids of humans and animals (6, 39). It was shown, that in addition to the well-known enterolignan precursors secoisolariciresinol and matairesinol, several other plant lignans were converted into enterolactone and enterodiol, although with varying degrees of efficiency (40). The health effects of lignans were evaluated in epidemiologic studies that used both the intake of secoisolariciresinol and matairesinol and plasma or urinary concentrations of enterolactone and enterodiol as exposure estimates. The calculated intake of secoisolariciresinol and matairesinol was based on published food composition tables (41–43), of which that provided by De Kleijn et al (41) is the most comprehensive. Of 8 published studies on enterolignan concentrations and the risk of CVD or cancer, only 2 measured enterodiol in addition to enterolactone. Enterolactone is usually measured with a time-resolved fluorimunoassay, which is not available for enterodiol (44).

**CVDs**

Two publications (45, 46) from one Finnish cohort study in which plasma enterolactone concentrations were studied in relation to CVD risk reported significant inverse associations (Table 3). In the Finnish Kuopio Heart Disease Risk Factor Study, a 65% lower risk of incident CAD was observed (46). With 2 additional years of follow-up monitoring, the risk of CAD death was of the same order of magnitude (RR: 0.44) and similar, although results were only borderline significant for total CVD deaths (RR: 0.55) (45). The association between serum enterolactone concentrations and plasma F2-isoprostane concentrations (a biomarker of in vivo lipid peroxidation) was studied cross-sectionally in a subset of 100 male participants in the Antioxidant Supplementation in Atherosclerosis Prevention Study (47). With higher enterolactone concentrations, F2-isoprostane concentrations were significantly lower (P = 0.02).

No studies on lignan intake and CVD risk have been published to date, but 2 cross-sectional studies related lignan intake to CVD risk factors. Of several risk factors studied among postmenopausal US women, only the waist-hip ratio (difference between extreme quartiles: −0.017; 95% CI: −0.030, −0.002; P for trend = 0.03) and the metabolic syndrome score, a summary score of several risk factors (difference between extreme quartiles: −0.55; 95% CI: −0.82, −0.28; P for trend = 0.0001), were associated with intake of secoisolariciresinol and matairesinol (48). Aortic stiffness, assessed with pulse-wave velocity measurements of the aorta, was borderline significantly inversely associated with lignan intake among postmenopausal Dutch women (49). The regression coefficient for those with a high intake of lignans was −0.41 (95% CI: −0.93, 0.11; P for trend = 0.06), compared with those with a low intake. The protective effect was most pronounced and significant among women with a postmenopausal time of > 20 y.

**Cancer**

To date, 3 prospective, nested, case-control studies and 3 case-control studies have studied plasma or urinary lignan concentrations and cancer risk (Table 4); all except one investigated breast cancer incidence. The 2 prospective, nested, case-control studies on breast cancer risk, among Dutch postmenopausal women (54) and among female participants in 3 cohorts in northern Sweden (53), found no relationship with plasma or urinary enterolactone concentrations. In contrast, all 3 case-control studies found an
**TABLE 2**
Prospective studies of flavonoid intake and risk of incident cancer and other chronic diseases

<table>
<thead>
<tr>
<th>Ref</th>
<th>Country</th>
<th>Population</th>
<th>Flavonoids</th>
<th>Comparison (high vs low intake)</th>
<th>Follow-up time</th>
<th>Outcome</th>
<th>No. of cases</th>
<th>Adjusted RR (high vs low)</th>
<th>P for trend</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>flavonoids</td>
<td>mg/d</td>
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<td></td>
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<td>F &gt; 29.9 vs &lt; 19</td>
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<td>Any cancer</td>
<td>75</td>
<td>1.21 (0.66, 2.21)</td>
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</tr>
<tr>
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<td>F &gt; 29.9 vs &lt; 19</td>
<td>5</td>
<td>Any cancer</td>
<td>57</td>
<td>1.02 (0.51, 2.04)</td>
<td>0.96</td>
</tr>
<tr>
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<td>&gt; 26.9 vs &lt; 4.3</td>
<td>30</td>
<td>Any cancer</td>
<td>1093</td>
<td>0.89 (0.74, 1.06)</td>
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</tr>
<tr>
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<td>F &gt; 39.5 vs &lt; 8.5</td>
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<td>5038</td>
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<td>&gt; 26.9 vs &lt; 4.3</td>
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<td>Any cancer</td>
<td>111</td>
<td>1.20 (0.71, 1.90)</td>
<td>0.51</td>
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<td>676</td>
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<td>151</td>
<td>0.53 (0.29, 0.97)</td>
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<td>1.20 (0.71, 1.90)</td>
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<td>&gt; 26.9 vs &lt; 4.3</td>
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<td>Any cancer</td>
<td>133</td>
<td>1.70 (1.00, 2.70)</td>
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<td>F &gt; 39.5 vs &lt; 8.5</td>
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<td>Any cancer</td>
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<td>0.97 (0.71, 1.32)</td>
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<td>&gt; 29.9 vs &lt; 19</td>
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<td>Any cancer</td>
<td>72</td>
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<td>Any cancer</td>
<td>132</td>
<td>0.55 (0.32, 0.95)</td>
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<td>&gt; 26.9 vs &lt; 4.3</td>
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<td>Any cancer</td>
<td>90</td>
<td>0.84 (0.43, 1.64)</td>
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<td>F &gt; 39.5 vs &lt; 8.5</td>
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<td>Any cancer</td>
<td>133</td>
<td>1.70 (1.00, 2.70)</td>
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<td>1.20 (0.73, 1.80)</td>
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<td>Any cancer</td>
<td>92</td>
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<td>F &gt; 39.5 vs &lt; 8.5</td>
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<td>Any cancer</td>
<td>54</td>
<td>0.84 (0.29, 2.45)</td>
<td>—</td>
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<td>&gt; 26.9 vs &lt; 4.3</td>
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<td>Any cancer</td>
<td>95</td>
<td>1.11 (0.61, 2.01)</td>
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</tr>
<tr>
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<td>F &gt; 39.5 vs &lt; 8.5</td>
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<td>Any cancer</td>
<td>226</td>
<td>1.30 (0.87, 1.80)</td>
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<td>&gt; 26.9 vs &lt; 4.3</td>
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<td>Any cancer</td>
<td>62</td>
<td>1.39 (0.56, 3.46)</td>
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</tr>
<tr>
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<td></td>
<td>F &gt; 39.5 vs &lt; 8.5</td>
<td></td>
<td>Any cancer</td>
<td>1069</td>
<td>1.04 (0.84, 1.28)</td>
<td>1.00</td>
</tr>
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<td>&gt; 26.9 vs &lt; 4.3</td>
<td>30</td>
<td>Any cancer</td>
<td>125</td>
<td>1.23 (0.72, 2.10)</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>F &gt; 39.5 vs &lt; 8.5</td>
<td></td>
<td>Any cancer</td>
<td>605</td>
<td>1.02 (0.72, 1.44)</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt; 26.9 vs &lt; 4.3</td>
<td>30</td>
<td>Any cancer</td>
<td>87</td>
<td>0.72 (0.36, 1.48)</td>
<td>—</td>
</tr>
</tbody>
</table>
inverse association between lignan concentrations and breast cancer risk (52, 55, 56). When a subset of postmenopausal women only was included in the analysis of the Shanghai Breast Cancer Study, the inverse association was no longer significant and the RR increased from 0.40 (95% CI: 0.24, 0.64) to 0.50 (95% CI: 0.23, 1.10) (51). In all of these case-control studies, plasma or urine was collected after diagnosis and sometimes even after initiation of treatment of the disease, which might have influenced lignan concentrations through changes in the diet as a result of disease or through other mechanisms. The only study on prostate cancer risk conducted to date found no association with plasma enterolactone concentrations among a large cohort of male residents of Finland, Norway, and Sweden (50).

Intake of the lignans secoisolariciresinol and matairesinol was studied in relation to the risk of several cancers in 1 prospective cohort and 3 case-control studies, all from the United States (Table 4). A significant inverse association was observed for breast cancer among premenopausal women (RR: 0.49) but not postmenopausal women (RR: 0.72) in western New York State (58). In contrast, breast cancer risk was borderline significantly elevated with a high intake of secoisolariciresinol and matairesinol among a large group of women participating in the multi-ethnic Bay Area Breast Cancer Study (RR: 1.3) (59) and of secoisolariciresinol only in the prospective California Teachers Study (RR: 1.4) (57). In the latter study, the association was substantially attenuated to a RR of 1.2 (95% CI: 0.9, 1.6) after adjustment for wine consumption. This led the authors to conclude that the increased risk with secoisolariciresinol was attributable to confounding by alcohol consumption.

Significant or borderline significant protective associations were also reported for endometrial cancer (60), ovarian cancer (37), and thyroid cancer (61) among female participants. No associations between lignan intake and incident prostate (34) or testicular (36) cancer were found.

### TABLE 2

**Continued**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Country</th>
<th>Population</th>
<th>Flavonoids</th>
<th>Comparison (high vs low intake)</th>
<th>Follow-up time</th>
<th>Outcome</th>
<th>No. of cases</th>
<th>Adjusted RR (high vs low)</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatoid arthritis</td>
<td>Knekt et al, 2002 (13)</td>
<td>Finland</td>
<td>Flavonols, flavones, flavanones</td>
<td>M &gt; 26.9 vs &lt; 4.3</td>
<td>28</td>
<td>CVD</td>
<td>103</td>
<td>0.55 (0.29, 1.01)</td>
<td>0.04</td>
</tr>
<tr>
<td>Type 2 diabetes mellitus</td>
<td>Knekt et al, 2002 (13)</td>
<td>Finland</td>
<td>Flavonols, flavones, flavanones</td>
<td>M &gt; 26.9 vs &lt; 4.3</td>
<td>28</td>
<td>CAD</td>
<td>70</td>
<td>0.44 (0.20, 0.96)</td>
<td>0.03</td>
</tr>
<tr>
<td>Cataracts</td>
<td>Knekt et al, 2002 (13)</td>
<td>Finland</td>
<td>Flavonols, flavones, flavanones</td>
<td>M &gt; 26.9 vs &lt; 4.3</td>
<td>28</td>
<td>Incident CAD</td>
<td>167</td>
<td>0.35 (0.14, 0.88)</td>
<td>0.01</td>
</tr>
<tr>
<td>Asthma</td>
<td>Knekt et al, 2002 (13)</td>
<td>Finland</td>
<td>Flavonols, flavones, flavanones</td>
<td>M &gt; 26.9 vs &lt; 4.3</td>
<td>28</td>
<td>382</td>
<td>0.65 (0.47, 0.90)</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

1 Flavonols: quercetin, kaempferol, myricetin; flavones: apigenin, luteolin; flavanones: hesperetin, naringenin, eriodictyol; catechins: (-)-catechin, (+)-gallocatechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate.

2 Mean, median, or category cutoff value.

3 Design in parentheses if other than prospective cohort.

4 95% CI in parentheses.

5 Quartiles were constructed for men and women separately, but RR is provided for sexes combined only.

**TABLE 3**

Prospective studies of serum lignans and risk of CVDs

<table>
<thead>
<tr>
<th>Reference</th>
<th>Country</th>
<th>Population</th>
<th>Lignan</th>
<th>Comparison (high vs low plasma concentration)</th>
<th>Follow-up time</th>
<th>Outcome</th>
<th>No. of cases</th>
<th>Adjusted RR (high vs low)</th>
<th>P for trend</th>
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</thead>
<tbody>
<tr>
<td>Vanharanta et al, 2003 (45)</td>
<td>Finland</td>
<td>1889 M</td>
<td>ENL</td>
<td>&gt; 23.9 vs &lt; 6.9</td>
<td>12.2</td>
<td>CVD</td>
<td>103</td>
<td>0.55 (0.29, 1.01)</td>
<td>0.04</td>
</tr>
<tr>
<td>Vanharanta et al, 1999 (46)</td>
<td>Finland</td>
<td>2005 M</td>
<td>ENL</td>
<td>&gt; 30.1 vs &lt; 7.2</td>
<td>10 (nested case-control)</td>
<td>CAD</td>
<td>70</td>
<td>0.44 (0.20, 0.96)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

1 ENL, enterolactone.

2 Design in parentheses if other than prospective cohort.

3 Death, unless indicated otherwise.

4 95% CI interval in parentheses.
DISCUSSION

In the past decade, several well-designed, prospective, cohort studies in which the health effects of flavonoids were studied have been published. The data regarding CVD suggest protective effects of high intakes of flavonols and flavones and possibly of catechins. However, only a few studies are available for catechins and for stroke; given the results obtained to date, these deserve more study. A meta-analysis of tea consumption in relation to CAD and stroke, including all studies published up to October 2000, was conducted by Peters et al (62). Most studies included in the current review were also included in that meta-analysis, because they provided data not only for tea, which is a major flavonoid source, but also for flavonoids. Peters et al (62) found evidence for publication bias, particularly with respect to stroke, and therefore urged caution in interpreting the results for this endpoint. Publication bias might have occurred for flavonoid epidemiologic studies as well. Another striking finding is that studies of CAD or myocardial infarction conducted in continental Europe reported strong inverse associations, whereas studies conducted elsewhere did not. Summarized RRs for drinking 3 cups per day compared with no tea were 1.62 (95% CI: 1.15, 2.30) for 2 studies from the United Kingdom, 0.27 (95% CI: 0.16, 0.44) for 3 studies from continental Europe, and 0.95 (95% CI: 0.84, 1.08) for 8 studies from the United States. Explanations for this phenomenon, which also seems to occur for flavonoids, include differing associations with a healthy lifestyle and publication bias. However, no satisfactory explanation has been provided, and research into these differences seems worthwhile.

Attempts to distinguish the effects of flavonols and flavones from those of catechins were undertaken with data from the Zutphen Elderly Study. Tea consumption was positively correlated with the intake of fruits and vegetables and their constituents, e.g., vitamin C, vitamin E, carotenoids, folate, and fiber. For the intake of vitamin C, β-carotene, and fiber, correlations in several European populations on the order of 0.40–0.70 were reported (63). When the correlation is too high, it is impossible to ascertain independent effects of dietary components, because of multicollinearity. This was the case for flavonoids and catechins in the Zutphen Elderly Study. Tea consumption was positively correlated with the intake of fruits and vegetables, some known and quantified, some less well characterized, and some unknown or unmeasurable. Many compounds tend to be present in the food contains a large number of different compounds, some

<table>
<thead>
<tr>
<th>Ref</th>
<th>Country</th>
<th>Population</th>
<th>Lignan</th>
<th>Comparison (high vs low)</th>
<th>Design</th>
<th>No. of cases</th>
<th>Adjusted RR (high vs low)</th>
<th>P for trend</th>
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<td>Scandinavia</td>
<td>3344 M</td>
<td>ENL</td>
<td>&gt; 15.6 vs &lt; 4.3</td>
<td>Nested case-control (3–24 y)</td>
<td>794</td>
<td>1.08 (0.83, 1.39)</td>
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<tr>
<td>Breast</td>
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<tr>
<td>Dai et al, 2003 (51)</td>
<td>China</td>
<td>234 F</td>
<td>ENL, END</td>
<td>— (urine)</td>
<td>Case-control</td>
<td>117</td>
<td>0.50 (0.23, 1.10)</td>
<td>0.09</td>
</tr>
<tr>
<td>Dai et al, 2002 (52)</td>
<td>China</td>
<td>500 F</td>
<td>ENL, END</td>
<td>— (urine)</td>
<td>Case-control</td>
<td>250</td>
<td>0.40 (0.24, 0.64)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hulten et al, 2002 (53)</td>
<td>Sweden</td>
<td>740 F</td>
<td>ENL</td>
<td>39.8 vs 5.3</td>
<td>Nested case-control (5–15 y)</td>
<td>248</td>
<td>1.1 (0.7, 1.7)</td>
<td>—</td>
</tr>
<tr>
<td>den Tonkelaar et al, 2001 (54)</td>
<td>Netherlands</td>
<td>356 F</td>
<td>ENL</td>
<td>96.9 vs 235.6 (urine, μmol/mol creatinine)</td>
<td>Nested case-control (9 y)</td>
<td>88</td>
<td>1.43 (0.79, 2.59)</td>
<td>0.25</td>
</tr>
<tr>
<td>Pietinen et al, 2001 (55)</td>
<td>Finland</td>
<td>402 F</td>
<td>ENL</td>
<td>&gt;34.8 vs &lt;6.2</td>
<td>Case-control</td>
<td>194</td>
<td>0.38 (0.18, 0.77)</td>
<td>0.03</td>
</tr>
<tr>
<td>Ingram et al, 1997 (56)</td>
<td>Australia</td>
<td>288 F</td>
<td>ENL</td>
<td>&gt;5250 vs &lt;1450 (urine, nmol/24 h)</td>
<td>Case-control</td>
<td>144</td>
<td>0.36 (0.15, 0.86)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>END</td>
<td>&gt;480 vs &lt;170 (urine, nmol/24 h)</td>
<td>Case-control</td>
<td>144</td>
<td>0.73 (0.33, 1.64)</td>
<td>0.29</td>
</tr>
<tr>
<td>Dietary intake</td>
<td>Prostate</td>
<td></td>
<td></td>
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<tr>
<td>Strom et al, 1999 (34)</td>
<td>US</td>
<td>190 M</td>
<td>SECO, MAT</td>
<td>&gt;0.48 vs &lt;0.48</td>
<td>Case-control</td>
<td>83</td>
<td>1.20 (0.65, 2.21)</td>
<td>0.55</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>&gt;0.05 vs &lt;0.05</td>
<td>Case-control</td>
<td>83</td>
<td>0.89 (0.47, 1.66)</td>
<td>0.71</td>
</tr>
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<td></td>
<td>Breast</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Horn-Ross et al, 2002 (57)</td>
<td>US</td>
<td>111 526 F</td>
<td>SECO, MAT</td>
<td>&gt;0.12 vs &lt;0.05</td>
<td>Cohort (2 y)</td>
<td>711</td>
<td>1.40 (1.00, 1.80)</td>
<td>0.02</td>
</tr>
<tr>
<td>McCann et al, 2002 (58)</td>
<td>US</td>
<td>617 F</td>
<td>SECO, END</td>
<td>393 F</td>
<td>premenopausal</td>
<td>301</td>
<td>0.49 (0.32, 0.75)</td>
<td>—</td>
</tr>
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<tr>
<td>Horn-Ross et al, 2001 (59)</td>
<td>US</td>
<td>2983 F</td>
<td>SECO, MAT</td>
<td>&gt;0.22 vs &lt;0.10</td>
<td>Case-control</td>
<td>1272</td>
<td>1.30 (1.00, 1.60)</td>
<td>—</td>
</tr>
<tr>
<td>Endometrial</td>
<td>Ovarian</td>
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<tr>
<td>Horn-Ross et al, 2003 (60)</td>
<td>US</td>
<td>942 F</td>
<td>SECO, MAT</td>
<td>&gt;0.24 vs &lt;0.12</td>
<td>Case-control</td>
<td>482</td>
<td>0.68 (0.44, 1.10)</td>
<td>0.03</td>
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<td></td>
<td>Thyroid</td>
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</tr>
<tr>
<td>McCann et al, 2003 (37)</td>
<td>US</td>
<td>820 F</td>
<td>SECO, MAT</td>
<td>&gt;0.71 vs &lt;0.30</td>
<td>Case-control</td>
<td>124</td>
<td>0.43 (0.21, 0.85)</td>
<td>&lt;0.05</td>
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<tr>
<td></td>
<td>Testicular</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walcott et al, 2002 (36)</td>
<td>US</td>
<td>295 M</td>
<td>SECO, MAT</td>
<td>&gt;1.42 vs &lt;0.28 μg/1000 kcal</td>
<td>Case-control</td>
<td>159</td>
<td>0.96 (0.11, 8.09)</td>
<td>0.27</td>
</tr>
</tbody>
</table>

1 ENL, enterolactone; END, enterodiol; SECO, secoisolariciresinol; MAT, matairesinol; —, no data provided.
2 Mean, median, or category cutoff value; plasma values in nmol/L, urine values as indicated, intake levels in mg/d.
3 Follow-up time in parentheses.
4 95% CI in parentheses.
5 ENL and END production from foods determined by in vitro fermentation with human fecal microflora.

TABLE 4
Prospective and case-control studies on plasma or urinary lignan concentrations or dietary lignan intake and incident cancer
supplied 87% of this population’s intake of catechins and 61% of the intake of flavonols and flavones (24). To circumvent multicollinearity problems but still distinguish the effects of catechins from those of flavonols, subgroups were defined, ie, tea, catechins from sources other than tea, and flavonols from sources other than tea. Independent effects on CAD mortality rates were borderline significant for tea \( (P = 0.06) \) and catechins from other sources than tea \( (P = 0.11) \). For correct interpretation of results of dietary component-based epidemiologic studies, adjustment for other dietary factors (both nutrient and nonnutrient) is of major importance.

Data are less convincing for cancer. Of several cancers studied, protective effects have been reported only for lung cancer in relation to flavonol and flavone intake. Together with data from one cohort study and one cross-sectional study suggesting beneficial effects on asthma and lung function, these data suggest a role for flavonoids in lung health that merits additional investigation. For colorectal cancer, data are inconsistent, with 1 positive, 1 inverse, and 4 null associations. Residual confounding by smoking is the most serious drawback of the flavonoid studies published to date. Unhealthy (or healthy) behaviors tend to cluster. Smoking, which is the single most important risk factor for many cancers and an important determinant of CVDs, is associated with higher intakes of energy, alcohol, and fat, lower intakes of fruits and vegetables, lower socioeconomic status, and physical inactivity (64–66). Previous studies showed that consumption of important sources of flavonoids, such as tea in the Netherlands (67) and in Japan (68) and wine in Denmark (69), is associated with healthy dietary patterns. Residual confounding occurs if confounders such as smoking are insufficiently accounted for in statistical analyses. Insufficient control for confounders can occur as a result of misclassification of the confounding factors, and control thus depends on the quality and amount of detail with which the confounders are measured. In particular, if the confounding is strong, as is usually the case for smoking, then misclassification of the confounder can yield spurious associations (70). Studying associations among lifelong nonsmokers is an effective way of ruling out residual confounding by smoking, and this should be done in future studies.

The strong inverse associations found for plasma enterolactone concentrations and the risk of CAD in a prospective study in Finland and supportive data from several cross-sectional studies make this an exciting new area of research that requires more investigation. Lignans are phytoestrogens, and their effect on breast cancer is a more traditional area of research which requires more investigation. Lignans are phytoestrogens, and their effect on breast cancer is a more traditional area of research that requires more investigation. Lignans are phytoestrogens, and their effect on breast cancer is a more traditional area of research that requires more investigation. Lignans are phytoestrogens, and their effect on breast cancer is a more traditional area of research that requires more investigation. Lignans are phytoestrogens, and their effect on breast cancer is a more traditional area of research that requires more investigation. 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48. McCann SE, Moutsik KB, Freudenheim JL, Ambroseon CB, Shields...


Risks and safety of polyphenol consumption

Louise I Mennen, Ron Walker, Catherine Bennetau-Pelissero, and Augustin Scalbert

ABSTRACT
This article gives an overview of the potential hazards of polyphenol consumption, as reported during the round-table discussion at the 1st International Conference on Polyphenols and Health, held in Vichy, France, November 2003. Adverse effects of polyphenols have been evaluated primarily in experimental studies. It is known, for example, that certain polyphenols may have carcinogenic/genotoxic effects or may interfere with thyroid hormone biosynthesis. Isoflavones are of particular interest because of their estrogenic activity, for which beneficial as well as detrimental effects have been observed. Furthermore, consumption of polyphenols inhibits nonheme iron absorption and may lead to iron depletion in populations with marginal iron stores. Finally, polyphenols may interact with certain pharmaceutical agents and enhance their biologic effects. It is important to consider the doses at which these effects occur, in relation to the concentrations that naturally occur in the human body. Future studies evaluating either beneficial or adverse effects should therefore include relevant forms and doses of polyphenols and, before the development of fortified foods or supplements with pharmacologic doses, safety assessments of the applied doses should be performed. Am J Clin Nutr 2005;81(suppl):326S–9S.

KEY WORDS Risk, safety, polyphenols

INTRODUCTION
In the past few decades, accumulating data have shown potential beneficial effects of polyphenols (1, 2). This has sometimes led to overestimation of the current knowledge regarding their effects, with disregard for the fact that some polyphenol-rich foods were previously considered to be inedible (soy, for example). These studies have stimulated additional research, focusing on the health effects of polyphenol-rich foods, specific phenolic compounds, or supplementation with a combination of several types of polyphenols. When extensive food composition tables for polyphenols become available, thorough observational epidemiologic studies can be carried out, potentially confirming the encouraging results of the mainly experimental data reported to date. Small-scale human intervention trials may even be planned to verify effects on surrogate endpoints of disease. Before we enter that stage, however, we must examine the potential adverse effects of polyphenols. With the disappointing results of the intervention trials with β-carotene supplementation (3) in mind, we need to consider the fact that polyphenols may, in specific populations, have effects opposite those we desire. In other words, the safety of elevated intakes cannot be assumed.

INTAKE
In the recommendations made by companies selling various nutritional supplements rich in polyphenols, some recommend the consumption of 50 mg/d isoflavones or 100–300 mg/d grape seed extracts rich in proanthocyanidins. These intake levels are close to those derived from the consumption of soy products in Japan or of grapes or wine in some European countries (4, 5). However, some supplement manufacturers recommend intakes far higher than those currently associated with the diet. Tablets or capsules containing 300 mg quercetin, 1 g citrus flavonoids, or 20 mg resveratrol, with suggested use of 1-6 tablets or capsules per day, are commonly found on the Internet. This would result in intakes ~100 times higher than the common intakes in a Western diet.

Furthermore, some of these supplements may appear safe when isolated from food plants, but the method of extraction used to produce the supplements may influence the nature of the compounds ingested and thus the safety of the product. This occurred with a hydroalcoholic extract of tea buds, sold as a slimming supplement, which was withdrawn from the market because of severe cases of liver toxicity (6).

RISK ASSESSMENT AND SAFETY EVALUATION
This takes us directly to the problem of risk assessment and safety evaluation. Hazard, risk, and safety are different issues, each of which should be considered (Table 1). A thorough risk assessment for polyphenols is complicated, not only because so many different compounds exist but also because not all necessary tools are currently available. Although hazards may be identified and characterized, no exposure assessment (ie, known/proposed intake) can be made, because of missing food composition data. Assessment of exposure through the measurement of biomarkers has also proved difficult, because metabolic...
TABLE 1
Terms used in risk assessments and safety evaluations

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Hazard</td>
<td>Potential for causing adverse effects</td>
</tr>
<tr>
<td>Risk</td>
<td>Probability that adverse effects will occur at a specified dose/level</td>
</tr>
<tr>
<td>Safety</td>
<td>Practical certainty that no adverse effects will be observed</td>
</tr>
</tbody>
</table>

specificity among populations and individuals may exist and techniques for simple measurements of such biomarkers and corresponding validity data are mostly lacking. It is therefore extremely difficult to know whether proposed intakes are safe or what the likely risks are with those intakes. Several undesirable effects of different phenolic compounds have been observed and are described here, as an example of hazard identification (Table 2). These were also discussed during the round-table discussion at the 1st International Conference on Polyphenols and Health, but the list of effects is not exhaustive.

HAZARD IDENTIFICATION FOR POLYPHENOLS

Most studies of polyphenols aimed to determine the protective effects of polyphenols against diseases or toxic drugs, and relatively few investigators have examined their possible toxicity. No acute toxicity was observed after oral administration of a grape seed proanthocyanidin extract at a dose of 0.5 or 2 g/kg body weight to rats or mice (7) or after administration of punicalagin (an ellagitannin present in pomegranate juice) at a dose of 60 g/kg diet to rats (8). However, chronic nephropathy was observed in rats when high doses of quercetin (2% or 4%) were added to their diet (9). No effect on survival times was observed in that study, whereas addition of quercetin (0.1%) to the diet of mice significantly reduced their life expectancy (10).

Some polyphenols may have carcinogenic or genotoxic effects at high doses or concentrations (11–13). Caffeic acid, for example, when present at a 2% level in the diet, induced forestomach and kidney tumors in rats and mice (14). Linear extrapolation of these data indicates appreciable risk at normal dietary levels. Furthermore, catecholstrogens are postulated to mediate induction of renal tumors by estradiol. Quercetin inhibits O-methylation of catecholstrogens and increases kidney concentrations of 2- and 4-hydroxyestriol by 60-80%. This may result in enhanced redox cycling of catecholstrogens and estradiol-induced tumorogenesis (15, 16). It is possible that the genotoxic effects observed in vitro may be attributable to the high concentrations used, at which polyphenols may become prooxidants (17). The formation of glutathionyl quercetin adducts has been shown in tyrosinase-rich B16F-10 melanoma cells and in the myeloperoxidase-rich human HL-60 cell line, which provides important evidence for the prooxidative metabolism of quercetin in cellular in vitro models (H van der Woude, personal communication at the 1st International Conference on Polyphenols and Health, 2003) (18). This also suggests that tissues rich in oxidative enzymes may be particularly vulnerable to the prooxidant toxicity of quercetin. Finally, green tea catechins (1% or 0.1% of the diet) have been found to enhance tumor development in the colon of F344 male rats and, although quercetin may decrease cancer cell proliferation at high doses, it has been found to stimulate cell proliferation at low doses (1–5 μmol/L) (19, 20).

In common with synthetic antioxidants, several flavonoids can inhibit thyroid peroxidase and interfere with thyroid hormone biosynthesis (free radical iodination) (21, 22). When vitexin, a C-glycosylflavone abundant in milklet, was administered to rats, it increased thyroid weight and decreased the plasma levels of thyroid hormones (23). This is thought to be one of the causes of endemic goiter in West Africa, where milklet is a staple food. Furthermore, a reduction of thyroid peroxidase activity was observed in rats fed a diet supplemented with genistin (24, 25). These effects of genistein on thyroid function are more pronounced in cases of iodine deficiency. This is of particular concern for babies exposed to particularly high doses of isoflavones through soy feeding (26). Among adults, however, 2 clinical studies failed to show significant effects on thyroid hormones after consumption of isoflavone-containing soy proteins for 3-6 mo (27, 28).

Isoflavones are a family of polyphenols that are distinctive because of their estrogen-like activity. It is because of this activity that they may have beneficial as well as adverse effects (29, 30). Total plasma isoflavone levels are generally between 0.05 and 5 μmol/L even in Asian populations, which represent consumers of large amounts of isoflavone-rich products such as soy. The intake from a Western diet is estimated as 0.2-5 mg/d, whereas a traditional Asian diet delivers 20-120 mg isoflavones/d (31, 32). These levels of intake were deemed safe in a comprehensive review, although sufficient data to draw conclusions regarding effects on cancer or neurologic diseases were lacking (33). High intakes have been associated with reduced fertility in animals and with anti-luteinizing hormone effects among premenopausal women (34–37). Furthermore, concerns have been expressed regarding sexual maturation of infants receiving very high levels of isoflavones in soy-based infant formula (38, 39). This is of particular importance for baby boys, who normally exhibit luteinizing hormone secretion between birth and 6 mo of age (40). It is therefore important to note that beneficial effects of isoflavones on the development of cancer through the inhibition of certain enzymes have been observed at levels that are all much higher (some >20 times higher) than those observed normally in human plasma (41–43). At these levels, isoflavones may have antiandrogenic effects, influence male and female fertility and sexual development in utero and after birth, and induce testicular atrophy (44–47).

Consumption of polyphenols may also have antinutritional effects. The inhibition of nonheme iron absorption attributable to simultaneous tea consumption is well known; high consumption of polyphenols may increase the risk of iron depletion in populations of individuals with marginal iron status (48). Important in this respect is the fact that major sources of polyphenols, such as coffee, tea, and wine, which are regularly consumed with meals, do not contain vitamin C, which is an enhancer of nonheme iron absorption (49). Furthermore, proanthocyanidins (condensed tannins) and ellagitannins have been considered antinutritional compounds, particularly in animal nutrition, because they are

TABLE 2
Hazards related to polyphenols

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<th>Hazard</th>
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<tbody>
<tr>
<td>Carcinogenicity/genotoxicity</td>
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<tr>
<td>Thyroid toxicity</td>
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<tr>
<td>Estrogenic activity of isoflavones</td>
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<tr>
<td>Antinutritional effects</td>
</tr>
<tr>
<td>Interactions with pharmaceuticals</td>
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</tbody>
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able to interact with proteins and inhibit several enzymes. They affected growth and digestibility in rats when added to the diet at a high dose (10 g/kg diet) but not at a lower dose (50). Consumption of proanthocyanidin-rich fava beans by Egyptian boys reduced the net protein utilization, which was restored with de-hulling of the beans (51). It should be noted that these particular effects are unlikely to occur with regular Western diets, which are characterized by a much lower tannin intake (52).

Finally, polyphenols may affect drug bioavailability and pharmacokinetics. Some drugs, such as benzodiazepines and terfenadine, show up to 3-fold increases in bioavailability with grapefruit juice (rich in naringenin), because of inhibition of CYP3A4 (53–55). These effects, which may be attributable in part to psoralens as well as naringenin, are clinically significant in the case of cyclosporine, because of a narrow therapeutic range (eg, when used after organ transplants).

Most of these effects have been shown in in vitro or animal studies, and it has not been proved that these effects also occur among humans. Intakes from habitual diets are usually lower than the doses used in these studies, and the food matrix may also influence the effects of polyphenols, which may explain why observational epidemiologic studies have not shown, for example, any carcinogenic effects of polyphenols to date (56) (although this is probably also attributable to lack of accurate exposure assessment and residual confounding). However, we must take the results of the experimental studies seriously, as seriously as we take the beneficial effects. Therefore, the known carcinogenic and endocrine system-disrupting effects of certain polyphenols in animals make human trials with high doses of these polyphenols unethical.

CONCLUSIONS

It is clear from the aforementioned findings that, in evaluations of experimental studies, we must look carefully at the doses used. In the 17th century, Paracelsus said, “All substances are poisons, there is none which is not a poison. It is the dose that distinguishes a poison from a remedy.” A dose that produces a beneficial effect in cell cultures may be poisonous when applied in a human setting. Alternatively, a dose used in an experimental study may never occur in a human setting, because consumption never reaches the same level, because the bioavailability is very low, or because the appropriate dose never reaches the target site. The form of the phenolic compound is also important, because phenolic compounds occur in food mainly as conjugated compounds and the substances occurring in plasma and tissues are mainly mammalian conjugates, except for certain isoflavones and flavonols. All of these aspects must be taken into account in the design of future experimental studies in the field of polyphenols; there is a need to try to model the human situation more closely, irrespective of whether studies are aimed at evaluating beneficial or adverse effects.

Finally, it must be pointed out that exposure levels depend on the mode of presentation of the polyphenols. The risk of consuming high doses of polyphenols from naturally polyphenol-rich foods is low, but we must take into account the negative effects of other ingredients in these foods, such as cholesterol-increasing fats in coffee, alcohol in wine, and fat in chocolate. Foods can be fortified with polyphenols, but we must be sure that they are consumed by the target populations for which they are designed and not by populations that are potentially at risk, such as children and pregnant women. Dietary supplements that contain high (ie, pharmacologic) doses of polyphenols can be developed. The intake of polyphenols may then easily reach very high levels; in such cases, toxicologic testing may be required to ensure safe levels of intake. In this respect, a recent report on the assessment of the safety of botanicals and botanical preparations for use in food and food supplements might very well apply to the field of polyphenols (57). The type of safety evaluation would depend on the nature of the polyphenol-containing product (a food, a food extract, or a pure compound) and on the proposed use potentially leading to a significant increase in exposure.

Before human intervention trials are designed to evaluate the effects of polyphenols on chronic diseases, with the use of fortified foods or supplements (with either nutritional or pharmacologic doses of polyphenols), a safety assessment of the applied dose should be performed, to prevent unethical studies from being conducted. Before we reach that stage, however, we need to accumulate substantial data from in vitro, animal, and observational epidemiologic studies with only relevant forms and doses, to ascribe a potential beneficial effect to total or specific polyphenol intake.

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ment of botanicals and botanical preparations for use in food and food 
Polyphenols: factors influencing their sensory properties and their effects on food and beverage preferences

Isabelle Lesschaeve and Ann C Noble

ABSTRACT
Bitterness and astringency are found in a variety of foods, including nuts, fruits, chocolate, tea, wine, and soymilk. In fruits and beverages, the taste of bitterness and the tactile sensation of astringency are elicited primarily by flavanol polymers (proanthocyanidins or condensed tannins). Variations in proanthocyanidin composition, such as polymer size, extent of galloylation, and formation of derivatives, affect both bitterness and astringency. In beverages, other factors also influence these sensations, including the pH and the levels of ethanol, sweetness, and viscosity. Similarly, foods eaten with beverages can influence astringency. For example, eating dark chocolate increases the astringency of red wine more than does milk chocolate. Individuals perceive astringency differently because of variations in salivary flow rates, and preferences for and acceptance of a product may vary tremendously among individuals; decreasing bitterness and/or astringency may not increase preference. Factors influencing bitterness, astringency, and individual preference decisions are discussed.

KEY WORDS Polyphenols, astringency, bitterness, sensory perception, consumer preference

INTRODUCTION
In the past decade, there has been growing interest in nutraceuticals and functional foods in Western countries. Many new products focused mainly on ingredient functionality and not on sensory properties. Consumers anticipated and accepted an unbalanced flavor, as long as the product delivered health benefits. In fact, many consumers thought that, if the product tasted bad, then it was obviously good for you. Consumers now want both health benefits and palatable food.

Flavanol polymers are being actively studied because of their cancer prevention properties and other potential nutritional benefits. However, their sensory properties can be summarized with 2 main descriptors, bitterness and astringency, which are well known for eliciting negative consumer reactions when present at high intensity. Factors influencing bitterness, astringency, and individual preference decisions are discussed here.

SENSORY PROPERTIES OF POLYPHENOLS
Compounds responsible
In beverages such as tea, cider, and red wine, as well as in several types of fruits, nuts, and chocolate, the taste of bitterness and the tactile sensation of astringency are elicited primarily by the flavonoid phenols, including flavans and flavonols. Of these, the flavan-3-ol monomers (catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate) and their oligomers and polymers, which are called proanthocyanidins or condensed tannins, are the most abundant in wine and tea. Both procyanidins (polymers of epicatechin and catechin) and prodelphinidins (polymers of epigallocatechin) have been detected in grapes (1, 2), whereas tea contains monomers and polymers of all 5 flavanols (3). With the exception of the bitterness of caffeine in tea, the flavanols are the primary sources of bitterness and astringency in tea and red wine.

Bitterness
Bitter taste is elicited by structurally diverse compounds, but no clear definition of the molecular properties that confer bitterness has been proposed (4, 5). Taste receptor cells are primarily associated with papillae on the tongue. Several transduction mechanisms for perception of bitterness have been identified and appear to be compound specific (6). Although the mechanisms through which bitter taste perception occurs are not well understood, they are the focus of intensive research, as reviewed elsewhere (7, 8).

Astringency
The tactile sensation of astringency is thought to be perceived through touch, via mechanoreceptors (9). Astringency is described sensorially as a puckering, rough, or drying mouth-feel, whereas an astringent is defined chemically as a compound that precipitates proteins. For water-soluble phenols, molecular weights between 500 and 3000 were reported to be required (10). Consistent with this definition, the assay for tannins developed by Adams and Harbertson (11) can detect only tannins that have > 3 flavan-3-ol units. Despite the inability of phenolic compounds with molecular weights of < 500 to precipitate proteins in chemical assays, flavan-3-ol monomers (12–14), flavan-3-ol dimers and trimers (15), and hydroxybenzoic acids (16) have been shown to elicit the sensation of astringency. Astringency of

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these smaller phenols may arise from formation of unprecipitated complexes with proteins (17) or cross-linking of proteins with simple phenols that have 1,2-dihydroxy or 1,2,3-trihydroxy groups, as proposed by McManus et al (18). Clifford (19) has provided an excellent review.

FACTORS AFFECTING THE SENSORY PROPERTIES OF POLYPHENOLS

Sensory methods

Evaluation of astringent products such as red wine or tea cannot be made in typical side-by-side comparisons. The intensity of astringency, as well as that of bitterness, builds up when several samples are tasted. Correspondingly, astringency increases with many sips of the same sample. Single-sip time-intensity protocols, in which the judge rates intensity from the time the sample is sipped until the sensation is no longer perceived, minimize carryover effects somewhat but do not reflect perception during normal consumption of beverages. The build-up of astringency has been illustrated in studies in which judges repeatedly sip astringent solutions, at defined intervals, while continuously rating astringency (20). As shown in Figure 1, the maximal intensity of astringency increased with repeated sipping of red wine at 25-s intervals (20). At each sip, astringency increased rapidly, reaching a maximum 6–8 s after the wine was swallowed. Intensity then decreased until the next sip was taken, whereupon another rapid increase occurred. However, increasing the time between sips to 30 s considerably reduced the enhancement of astringency with the second sip (21). A similar trend was observed when a significant increase in astringency occurred when red wines were sipped at 20-s intervals, whereas the increase in astringency with 40-s intervals was not significant (22). Astringency could continue to build during 8 sips, as shown for tea.

With each successive sip was not significant after the third sip, suggesting that a plateauing effect might have occurred by sip 3, in contrast to red wines, for which the increases for 4 sips were significant (20).

Chemical structures

Astringency increases and bitterness decreases with the mean degree of polymerization. In addition, small differences in flavonoid configurations can produce significant differences in sensory properties. Epicatechin is more bitter and astringent than its chiral isomer catechin (12, 14). Similarly, the bond location and the identity of the monomeric units influenced the astringency and bitterness of synthesized dimers and trimers (Figure 2). Although the trimers and 2 of the dimers were more astringent than the monomers, dimer B6 (catechin-4,6-catechin) was more bitter and astringent than dimer B3 (catechin-4,8-catechin) and dimer B4 (catechin-4,8-epicatechin) (15).

Vidal et al (23) studied the effects of tannin structure on bitterness and astringency. They showed that modifying the molecular structure by introducing an ethyl bridge decreased astringency but increased bitterness. In contrast, for grape seed tannin, reducing the level of esterification with gallic acid only decreased astringency.

Interactions in mixtures

Increasing the ethanol concentration of wine from 8% to 14% (by vol) approximately doubled the bitterness intensity but had no effect on astringency (24, 25). Ethanol enhancement of bitterness was also observed with model solutions (26). Lowering the pH of wines increased sourness but had no effect on bitterness (27). In contrast, adding acid (and concomitantly lowering the pH) has been reported to increase the astringency of cranberry juice (28), wines (25, 29, 30), and phenolic compounds in model solutions (25, 29–31).

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**FIGURE 1.** Average time-intensity curves for astringency of red wines (10 mL wine sipped at 25-s intervals). Adapted with permission from reference 20.
Typically, sweetness and bitterness are mutually suppressed in mixtures (32, 33). Independently increasing sweetness or viscosity decreased the intensity of bitterness in vermouths (34). Adding aspartame to model solutions of grape seed tannin decreased bitterness but had no affect on astringency (35). Conflicting with this, the addition of aspartame to red wine (36) decreased astringency significantly, as did the addition of sucrose (37). When grape seed tannin, citric acid, and alum were thickened with carboxymethylcellulose, astringency was significantly reduced with a viscosity increase of 5 cp (35, 38) (Figure 3). Similar reductions in astringency were demonstrated in carboxymethylcellulose-thickened alum solutions (39), cranberry juice (28), and soymilk (40).

Very little work has addressed the effects on astringency arising from interactions between food and wine. In one study, the astringency of 3 red wines increased after chocolate was tasted, but the increase was much larger after dark chocolate was sampled than after milk chocolate (Figure 4) (A Svensson, unpublished information). In a separate study, the sourness and bitterness of chardonnay wines decreased slightly after hollandaise sauces were tasted, with a larger effect being observed with a higher-fat sauce (41). Interestingly, both the chocolate and hollandaise sauce had larger effects on the wine flavor than the wine had on the flavor of the foods.

**PHYSIOLOGIC FACTORS**

**Propylthiouracil/phenylthiocarbamide status**

The wide range of individual sensitivities to the bitter compounds phenylthiocarbamide and propylthiouracil has been extensively documented (42). Individuals classified as propylthiouracil tasters perceive the bitterness of propylthiouracil more intensely, have a higher density of fungiform taste papillae, and have more taste pores per taste bud than do nontasters (42–44). Bartoshuk (45, 46) reported a trimodal distribution of responses to propylthiouracil, which she used to classify subjects as supertasters, tasters, or nontasters. Tasters were reported to be more sensitive to caffeine and quinine than nontasters (47, 48). Despite the large differences in genetically determined sensitivities to propylthiouracil (or phenylthiocarbamide), propylthiouracil status did not affect perceptions of the astringency of wine (37), acids (49), soymilk (40), or alum (50) or of the bitterness and astringency of phenolic compounds in water (14, 16, 31, 35). In contrast, Pickering et al (50) reported that supertasters and tasters were more sensitive than nontasters to the bitterness and astringency of red wine.

**Saliva**

Oral manipulation or even sipping of water can increase the salivary flow rate; however, dry crackers, acids, and astringents are the most potent elicitors of saliva flow. Unilateral monitoring of the salivary flow of the parotid salivary gland while subjects sipped wine and expectorated at 10 s revealed that the increase in flow rate was rapid but peaked at ~20 s and rapidly decreased until additional stimulation occurred (24). When subjects were partitioned into groups on the basis of their salivary flow rates and the data were analyzed separately, low-flow subjects perceived the maximal intensity of astringency later and rated it more intensely and for a longer time than did high-flow subjects, for both red wine.

**FIGURE 2.** Maximal intensities of astringency and bitterness for 2 flavan-3-ol monomers (catechin [CAT] and epicatechin [EPI]), 3 dimers (B3, B4, and B6), and 2 trimers (C2 and C) (0.9 g/L). Adapted with permission from reference 15.

**FIGURE 3.** Effects of increased viscosity on maximal astringency intensity of citric acid (1.2 g/L), alum (1 g/L), and grape seed tannin (GST) (2.5 g/L). Adapted with permission from references 35 and 38.
(37) and white wine (25) (Figure 5). However, conflicting results were observed in other studies, where astringency perception did not vary with salivary flow status (31, 35, 40).

When saliva flow is stimulated, saliva pH increases and the protein concentration decreases, although the total protein content remains fairly constant (51). Twenty-three percent of saliva proteins are basic proline-rich proteins (52), which have a strong affinity for binding polyphenols (53). HPLC profiles of proteins in human saliva revealed decreases in some proteins after ingestion of wine and grape seed tannin solutions, presumably as a result of tannin binding to these proteins. The appearance of a new protein peak 8 min after tasting was postulated to indicate the formation of a soluble protein-tannin complex (54). The oral sensation of astringency of polyphenolic compounds is thought to be linked to precipitation of these salivary proteins. It has been speculated that astringency is the friction perceived when oral lubrication is reduced after binding of astringent compounds to salivary proteins (35, 55, 56). Therefore, the lower astringency ratings for the high-flow subjects in Figure 5 may reflect their greater ability to restore oral lubrication, as a result of their greater volume of saliva.

Effects of bitterness and astringency on preferences

Taste preferences for sweet solutions and sweet products have been extensively studied, but there are far fewer studies addressing acceptance of bitterness or astringency. Bitter beverages (eg, beer, coffee, and tea) are not well accepted by children. Bitter beverages have been shown to be rejected because of their taste; beer and strong black coffee were rejected by 94% and 90% of American students (19–31 y), respectively (57). Astringency, like bitterness, is often perceived as a negative attribute, such as in soy products (58), dairy products (59), nuts (60), and juices (61). The astringency and bitterness of many vegetables and fruits containing phytonutrients are often cited as the reason for consumers rejecting the plant products, despite their known health benefits (62).

It is worth noting that consumers do not always describe bitter and astringent perceptions with the expected descriptors. Recently, Lesschaeve (I Lesschaeve, unpublished observations, 2003) studied the relationship between the consumer language used to express likes and dislikes and sensory descriptors of red wines. When consumers tended to like the wines, they did not use bitter as a descriptor; bitter was used to express dislike and tended to be associated with acid and astringent sensory characteristics, not bitterness. In the same study, consumers who liked astringent wines described them as having “a lot of character” or “a long aftertaste.”

When products are consumed under normal conditions, the sample is repeatedly ingested, which results in an increase in the intensity of both bitterness and astringency, as discussed above. Only one study has addressed the impact of this phenomenon on
consumer liking (63). The temporal changes in bitterness, astringency, and overall aroma that occurred with repeated sips of wine influenced consumers’ liking. However, the hedonic responses varied among the consumer segments and wine styles. Among both frequent and less-frequent consumers of wine, some individuals liked the wine more as astringency increased, whereas others had a negative response to the increase in astringency.

**Effects of exposure, social influences, and extrinsic factors on preferences**

Preferences change with time, as noted for textural attributes of food by Szczesniak (64). Children show a developmental pattern of preferences for food textures, progressing from soft, smooth, unidimensional textures to firm, rough (astringent), complex textures. Acquisition of liking for innately disliked products is possible. With even short-term exposure to bitter foods, hedonic ratings increased (65, 66). In experiments conducted with children (67, 68) or with adults (69), liking for novel food products increased after “forced” exposures. Pliner (69) suggested that, with the repeated exposure, the subjects had overcome their neophobia, ie, fear or reluctance to ingest novel foods. Stein et al (66) reported that a positive liking shift appeared after 7 days of exposure to a bittersweet drink, which was speculated to have occurred through learned association of flavor with postigestive consequences. In a study of several novel foods, Mattes (65) found that liking of bitter and sour foods was more resistant to change than were hedonic ratings for sweet and salty items.

Modifying the bitterness and/or astringency of foods by adding masking agents or developing debittering processes can enhance palatability. Such processes involve teaming with flavor companies, which recommend first working with a base to mask off-notes and then accentuating desired flavors with the addition of actual flavors. Working with a base to cleanse the product of undesired qualities helps avoid the problem of “overflavoring” and allows the flavorist to select the most complementary flavors for the formula (70).

Overall acceptability is also influenced by extrinsic properties, such as health claims, price, appearance of the label, brand, and color of the product. Moreover, the hedonic response is affected by the consumer’s expectations for the product, which are based on factors such as previous experience, peer pressure, expert recommendations, and brand familiarity (71, 72).

Social factors may be the most potent means of enhancing liking among humans. If an adult demonstrates pleasure when consuming a food, then this positive response can influence the hedonic response of a child (73). For many drinks, such as coffee, tea, beer, aperitif, and wine, the positive value generally associated with the social context of consumption can be an important mechanism for learning to like the beverage, which might have been unpalatable initially.

**CONCLUSIONS**

Many phenolic compounds with important health benefits are characterized by bitterness and astringency, which are often perceived as aversive. The intensity of these sensations can be modified with additives, such as sweeteners, or through modification of the concentrations or compositions of the polyphenols. Modifying the sensory properties may not be the only solution, because consumers very often like foods or beverages that are initially perceived as unpalatable. Learning to like astringent red wines may require repeated exposure and is enhanced by peer pressure and consumption under positive conditions.

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