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Do n−3 fatty acids prevent osteoporosis?1,2

Chaim Vanek and William E Connor

The essential polyunsaturated fatty acids (PUFAs) comprise 2 main classes: n−6 and n−3 fatty acids. The most common source of n−6 fatty acids is linoleic acid (LA), which is found in high concentrations in various vegetable oils. Arachidonic acid (AA), the 20-carbon n−6 fatty acid, is obtained largely by synthesis from LA in the body. The n−3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are found in fish and fish oils. The beneficial health effects of these two n−3 fatty acids were first described in the Greenland Eskimos, who consumed a high-seafood diet and had low rates of coronary heart disease, asthma, type 1 diabetes mellitus, and multiple sclerosis. Since that observation, the positive health attributes of n−3 fatty acids have been extended to include benefits related to cancer, inflammatory bowel disease, rheumatoid arthritis, and psoriasis (1, 2).

Both n−3 and n−6 fatty acids are incorporated into cellular phospholipid membranes, where they serve as precursors to the production of eicosanoids, such as prostaglandins, leukotrienes, and thromboxanes. Eicosanoids, which are derived from n−6 fatty acids, are less atherogenic, proinflammatory, and vasoconstrictive than the eicosanoids from n−3 fatty acids. For example, prostaglandin E2, thromboxane A2, and leukotriene B4 are derived from AA by cyclooxygenase and lipooxygenase enzymes and are well-described mediators of platelet aggregation, immune response, and vasoreactivity. Conversely, EPA is metabolized by cyclooxygenase and lipooxygenase to prostaglandin E3 and leukotriene B3, which are lesser promoters of platelet aggregation and immune reactivity (1). Dietary supplementation with n−3 fatty acids also reduces the production of interleukin 1 and tumor necrosis factor in response to an endotoxin stimulus (3). Therefore, a diet high in n−3 fatty acids favorably modulates the production of eicosanoids and cytokines that play a deleterious role in heart disease, cancer, and autoimmune diseases.

The study by Höggström et al (4) in this issue of the Journal nicely adds to a growing body of evidence that n−3 fatty acids are also beneficial to bone health. Höggström et al determined the fatty acid composition of the serum phospholipid fraction of 78 healthy young men and found a positive correlation between the n−3 fatty acid content and bone mineral density (BMD). BMD at the spine at age 22 y and changes in spinal BMD between the ages of 16 and 22 y were significantly correlated with higher serum concentrations of total n−3 fatty acids and DHA. Also, a negative association was found between higher ratios of n−6 to n−3 fatty acids and spinal BMD accrual between ages 16 and 22 y. A negative association between higher ratios of n−6 to n−3 fatty acids and BMD was also found in an earlier study of elderly men and women (5). However, the study by Höggström et al is unique in that it measured serum fatty acid concentrations rather than use a dietary-recall questionnaire to determine fatty acid intakes.

Animal models have suggested that n−3 fatty acids may attenuate postmenopausal bone loss. Ovariectomized mice fed a diet high in fish oil had significantly less bone loss at the femur and lumbar vertebrae than did ovariectomized mice fed a diet high in n−6 fatty acids (6). In vitro models using a preosteoblastic cell line, MC3T3-E1, indicated a greater production of the bone-formation markers alkaline phosphatase and osteocalcin after 48 h of treatment with EPA than after treatment with AA (7).

An additional mechanism of postmenopausal bone loss involves the findings that 1) increased marrow adiposity accompanies osteoporosis in aging populations (8) and 2) osteoblasts are derived from pluripotent mesenchymal stem cells that can differentiate into mature osteoblasts or adipocytes (9). An important regulatory mechanism controlling this differentiation is the peroxisome proliferator–activated receptor γ (PPARγ) nuclear transcription factor. PPARγ is a member of the nuclear receptor superfamily of proteins that mediates ligand-dependent transcriptional activation and repression. This protein governs genes that regulate metabolic functions, such as lipogenesis, fatty acid oxidation, and glucose uptake. PPARγ is expressed in adipose tissue and bone marrow.

Heterozygous PPARγ-deficient mice created by gene targeting exhibited enhanced osteoblastogenesis, increased bone volume, and decreased adipogenesis in femoral histology, radiology, and bone marrow stem cell cultures (10). Mice fed rosiglitazone, the PPARγ agonist, showed reduced bone mass, decreased osteoblasts, and increased marrow adiposity (11). Elderly women with type 2 diabetes who were taking PPARγ agonist (eg, troglitazone, pioglitazone, and rosiglitazone) anti-diabetic medication had greater bone loss at the lumbar spine than did those who were not taking this class of medication (12). Importantly, the n−6 polyunsaturated fatty acids can also activate PPARγ (13).

LA and AA are oxidized by lipooxygenases to form 13-hydroxyoctadecadienoic acid (13-HODE) and 15-hydroxyecosatetraenoic acid (15-HETE), respectively. 13-HODE and 15-HETE were the eicosanoids from n−6 fatty acids (6). In vitro models using a preosteoblastic cell line, MC3T3-E1, indicated a greater production of the bone-formation markers alkaline phosphatase and osteocalcin after 48 h of treatment with EPA than after treatment with AA (7).

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are known activators of PPARγ (13). In contrast, lipoxygenases convert EPA and DHA into 15-hydroxyeicosapentaenoic acid and 17-hydroxydocosahexanoic acid, which are not known activators of PPARγ. Overexpression of the Alox15 gene in mice, a lipoxygenase that converts LA and AA into 13-HODE and 15-HETE, results in decreased femoral BMD and femoral failure load (14). Allelic variations in the human lipoxygenase homologue, ALOX12 and ALOX15 on chromosome 17, are linked to variations in spinal BMD (15, 16).

Lowering the dietary ratio of n–6 to n–3 fatty acids may diminish PPARγ activation and thereby promote osteoblastogenesis at the expense of bone marrow adiposity. Unfortunately, the few studies that have been conducted to determine the effects of PUFAs on the prevention or treatment of osteoporosis have been contradictory (17). Sixty-five postmenopausal women receiving PUFA supplements (60% LA, 8% γ-linolenic acid, 4% EPA, and 3% DHA) had statistically significant increases in lumbar spine and femoral neck BMD compared with placebo. However, a study of 42 postmenopausal women receiving a similar mixture of PUFA supplements showed no significant increases in BMD compared with women receiving placebo. Both studies used high amounts of n–6 fatty acids (LA and γ-linolenic), which may have interfered with the outcomes.

n–3 Fatty acids play an important role in health and disease (2) and are thought to favorably affect skeletal health as well. The diseases prevented or ameliorated by n–3 fatty acids are as follows: coronary heart disease, stroke, autoimmune disorders, inflammatory bowel disease, osteoporosis, and cancers of the breast, colon, and prostate. The attainment of peak bone mass in adolescence and prevention of age-related osteoporosis are potential positive effects of n–3 fatty acids. Further elucidation of the physiologic effects of n–3 fatty acids on bone health, along with clinical trials of EPA and DHA to prevent or treat osteoporosis, is needed. 

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REFERENCES

The urgent need to recommend an intake of vitamin D that is effective1,2


The report by Hyppönen and Power in this issue of the Journal (1) highlights a frustrating and regrettable situation for nutrition researchers. In the early 1970s, the same serum 25-hydroxyvitamin D [25(OH)D] concentrations reported by Hyppönen and Power were thought to be indicative of “healthy” white adults in the United Kingdom (2). However, during those early years after the discovery of 25(OH)D, the adequacy of its serum concentration was based simply on whether the concentration was enough to prevent osteomalacia or rickets. Three decades later, we know that 25(OH)D concentrations relate to many other aspects of health, including fracture risk, bone density, colon cancer, and even tooth attachment (3); we also know that much higher concentrations of 25(OH)D are needed to prevent adverse outcomes. Indeed, in the 1958 British birth cohort, lower 25(OH)D is associated with a higher percentage of hemoglobin A1C (a measure of long-term glucose concentration), which further emphasizes the need to maintain optimal 25(OH)D concentrations (4).

Randomized trials using the currently recommended intakes of 400 IU vitamin D/d have shown no appreciable reduction in fracture risk (3). In contrast, trials using 700–800 IU vitamin D/d found less fracture incidence, with and without supplemental calcium (3). The reduction in fracture incidence occurs when mean serum 25(OH)D concentrations exceed 72 nmol/L, and this change may result from both improved bone health and reduction in falls due to greater muscle strength (3). Although it is not yet proven through clinical trials, higher intakes may also reduce the incidence of colon and other cancers, and these relations indicate that the desirable 25(OH)D concentration is ≥75 nmol/L (3).

One recent report associates greater 25(OH)D concentrations with lower risk of nursing home admission; the most desirable category of concentration starts at 75 nmol/L (5).

Human diets do not provide sufficient vitamin D; if they did, the abovementioned associations between health and serum 25(OH)D concentrations would not be so routinely observed. The vitamin D provided by foods and supplements is overwhelmed by the effect of skin exposure to ultraviolet B light. Geography, season, skin color, and sun-related behavior are the main predictors of vitamin D nutritional status (6–10). Correction of low 25(OH)D concentrations can happen only if some or all of the following are implemented: the encouragement of safe, moderate exposure of skin to ultraviolet light; appropriate increases in food fortification with vitamin D; and the provision of higher doses of vitamin D in supplements for adults.

Evaluation of most relations of health and disease that involve vitamin D leads to the conclusion that a desirable 25(OH)D concentration is ≥75 nmol/L (30 ng/mL) (3–5). If a concentration of 75 nmol/L is the goal to be achieved by consumption of vitamin D, then why is it so rare for members of the population to accomplish this? One reason is that almost every time the public media report that vitamin D nutrition status is too low, or that higher vitamin D intakes may improve measures of health, the advice that accompanies the report is outdated and thus

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and 600 IU for those aged 51–70 y. Some experts say that optimal amounts are closer to 1000 IU daily. Until more is known, it is wise not to overdo it.” The only conclusion that the public can draw from this is to do nothing different from what they have done in the past.

Supplemental intake of 400 IU vitamin D/d has only a modest effect on blood concentrations of 25(OH)D, raising them by 7–12 nmol/L, depending on the starting point. To raise 25(OH)D from 50 to 80 nmol/L requires an additional intake of ≈1700 IU vitamin D/d (11). Safety is the first priority when giving advice to increase supplementation or fortification with any nutrient. A recent review in this Journal applied the risk assessment method used by the Food and Nutrition Board to update the safe tolerable upper intake level (UL) for vitamin D (12). The method focuses on the risk of hypercalcemia. The conclusion was that the UL for vitamin D consumption by adults should be 10 000 IU/d (12). This indicates that the margin of safety for vitamin D consumption for adults is >10 times any current recommended intakes.

The balance of the evidence leads to the conclusion that the public health is best served by a recommendation of higher daily intakes of vitamin D (3). Relatively simple and low-cost changes, such as increased food fortification or increasing the amount of vitamin D in vitamin supplement products, may very well bring about rapid and important reductions in the morbidity associated with low vitamin D status. The current UL is but one impediment to this action; another is the perpetuation of outdated intake recommendations.

It is important for major journals such as the AJCN to publish evidence of a widespread nutrient deficiency. Regrettably, we are now stuck in a revolving cycle of publications that are documenting the same vitamin D inadequacy (1–3, 5, 7–9, 13–17). This phenomenon has been referred to as “circular epidemiology” (18), and, for vitamin D, the phenomenon will continue for as long as the levels of vitamin D fortification and supplementation and the practical advice offered to the public remain essentially the same as they were in the era before we knew that 25(OH)D even existed. As scientists, the purpose of our work is to improve the health of the public. We know the realities of serum 25(OH)D concentrations in populations around the world, and we have come to the conclusion that public health will benefit from improved vitamin D nutritional status. We know the intakes of vitamin D needed to bring about desirable 25(OH)D concentrations, so why is the science not making a difference to public health? A major reason is that there is little public pressure on policy makers to support efforts to update recommendations about nutrition. Public pressure is generally rooted in the media, but we do not think that the public media present the vitamin D story in a complete and accurate manner. Reports about vitamin D inadequacies are presented straightforwardly, but, when it comes to discussing the intake of vitamin D needed to correct the situation, outdated official recommendations for vitamin D are propagated by the public media. This probably occurs because of restrictive editorial policies driven by concern about possible litigation if media were to advise a “toxic” intake greater than the UL. The unfortunate result is that there is minimal motivation for policy makers to implement the relatively simple steps that could correct this nutrient deficiency.

Because of the convincing evidence for benefit and the strong evidence of safety, we urge those who have the ability to support public health—the media, vitamin manufacturers, and policy makers—to undertake new initiatives that will have a realistic chance of making a difference in terms of vitamin D nutrition. We call for international agencies such as the Food and Nutrition Board and the European Commission’s Health and Consumer Protection Directorate-General to reassess as a matter of high priority their dietary recommendations for vitamin D, because the formal nationwide advice from health agencies needs to be changed.

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REFERENCES

Liquid calories, sugar, and body weight\textsuperscript{1–3}

Adam Drewnowski and France Bellisle

\textbf{ABSTRACT}

The consumption of sugar-sweetened beverages has been linked to rising rates of obesity in the United States. The standard explanation is that energy-containing liquids are less satiating than are solid foods. However, purely physiologic mechanisms do not fully account for the proposed links between liquid sugar energy and body weight change. First, a reevaluation of published epidemiologic studies of consumption of sweetened beverages and overweight shows that most such studies either are cross-sectional or are based on passive surveillance of temporal trends and thus permit no conclusions about causal links. Second, research evidence comparing the short-term satiating power of different types of liquids and of solids remains inconclusive. Numerous clinical studies have shown that sugar-containing liquids, when consumed in place of usual meals, can lead to a significant and sustained weight loss. The principal ingredient of liquid meal replacement shakes is sugar, often high-fructose corn syrup, which is present in amounts comparable to those in soft drinks. Far from suppressing satiety, one such liquid shake is marketed on the grounds that it helps control hunger and prevents hunger longer when consumed for the purpose of weight loss. These inconsistencies raise the question whether the issue of sugars and body weight should continue to beframed purely in metabolic or physiologic terms. The effect of sugar consumption on body weight can also depend on behavioral intent, context, and the mode of use, availability, and cost of sweetened liquids. \textit{Am J Clin Nutr} 2007;85:651–61.

\textbf{KEY WORDS} Sweetened beverages, meal replacement shakes, weight gain, weight loss, satiety, cost

\textbf{INTRODUCTION}

Regular consumption of sugar calories in liquid form is said to be responsible for body weight gain (1–4). That is the conclusion of some epidemiologic and experimental studies that have linked the consumption of sweetened beverages in the United States to the rising rates of obesity and overweight (2, 5, 6). Sugar-sweetened beverages are said to promote obesity by virtue of their low satiety and high added sugar content (4).

In evidence-based medicine, one of the criteria for establishing causality is a biologically plausible mechanism (7). The reported links between sweetened beverage consumption and weight gain (4) rest largely on temporal parallels (1, 3) and cross-sectional studies (8). The similarity in time trends between growing beverage consumption (9, 10) and rising obesity rates in the United States (11–13) is indeed striking and cannot be denied. However, temporal associations are confounded by myriad factors, including dietary and secular trends, and by more sedentary lifestyles (14). Cross-sectional studies, based on a single point in time, do not allow the drawing of conclusions about causal links between sugar intake and the dynamics of weight change.

The search for a biologically plausible mechanism has come to focus on the notion that liquid calories are not perceived by the body. Despite the fact that short-term satiety signals may have little to do with the long-term homeostatic mechanisms regulating body weight, putative satiety deficits are routinely invoked to bolster associations found in epidemiologic studies (1, 4, 6, 15, 16). In fact, the entire debate about beverages and body weight gain has been framed in physiologic terms, and much attention has been paid to satiety (4, 15, 16), energy compensation (4, 17), glycemic index (18), and the vitamin and micronutrient content of beverages (6). When it comes to soft drinks consumption and body weight change, most researchers have opted to implicate human physiology (1, 4, 6, 15, 16, 18) rather than to explore human dietary behavior or the economics of food choice.

Sugar-containing beverages include still and carbonated soft drinks, juice-based beverages, 100% juices, and flavored milk. The overwhelming emphasis has been on soft drinks, and beverages sweetened with high-fructose corn syrup (HFCS) rather than sucrose have come under particular scrutiny (1, 3). In animal models, the consumption of pure fructose or a fructose-rich diet is reported to suppress insulin secretion and leptin production (19, 20), thereby promoting weight gain. One hypothesis is that fructose, whether consumed in solid or in liquid form, does not stimulate insulin secretion or leptin production in humans and may potentially contribute to weight gain (21). However, the question of whether HFCS promotes human obesity (1) requires further study (4). This review will distinguish between different...
types of sweetened beverages by using the terms and definitions provided by the authors of the studies referenced. Despite its popularity, the notion that liquid calories are not perceived by the body (17, 22) rests on inconclusive evidence. As documented in a review, some studies showed that liquids were less satiating than were solids, whereas other studies showed the exact opposite (23). In one study, jelly beans led to energy compensation, but beverages did not (19); in another study, cookies and cola had identical effects on hunger ratings and energy intakes (EIs) at lunch (24). The argument that humans are ill-adapted to liquids (17) has now been extended to some solid foods (21, 25). Solid fast foods of high energy density are reported to have low satiating power (25).

The notion that the consumption of sugared beverages must result in weight gain (17, 22) runs counter to a large body of clinical literature on the consumption of sugared beverages for the purpose of weight loss (26). Not everyone fully appreciates that the principal ingredient of liquid meal replacement (MR) shakes is sugar, which is present in quantities comparable to those in caloric soft drinks (36–72 g sugar/d). As the name implies, liquid MR shakes are sugared beverages consumed for the specific purpose of weight loss. In randomized clinical trials with overweight adults, daily consumption of liquid MR shakes, some containing HFCS, led to a sustained and significant weight loss (26, 27). Sugared liquid shakes were reported to be more effective in promoting weight loss than were low-fat diets that included plenty of vegetables and fruit (26).

Clearly, additional insights should come from studies in behavior and the economics of food selection. Depending on who uses them, in what context, and for what purpose, sugar-containing liquids can lead either to weight gain or to weight loss (26). Price may be one issue (28). Sweetened beverages are the largest source of inexpensive added sugars in the US diet (28). Liquid MR shakes provide the same sugar energy as do sugar-sweetened beverages, but for different population subgroups, in a different context, and at a higher cost. The links between sugar consumption and body weight change may well depend on the purpose, context, and mode of use of liquid sugar calories and on the beverages’ availability and price. A critical reexamination of the role of liquid sugar energy in weight control is the focus of this report.

SUGARS AND BODY WEIGHT

The consumption of sweetened beverages in the United States has increased sharply over the last 2 decades (9, 10). Analyses of the data from the third National Health and Nutrition Examination Survey (NHANES III; from 1988–1994) showed that beverages (including milk) provided 20%–24% of dietary energy across all age groups, and that soft drinks accounted for 8% of energy in the adolescents’ diet (8). By 2001, soft drinks accounted for 9.2% of daily energy intake in persons >2 y old, and another 7.3% of energy was supplied by fruit juices and milk (10). Analyses of the 1994–1996 Continuing Survey of Food Intake by Individuals (CSFII) data found that added sugars accounted for 15.8% of daily EIs; more than one-third of sugar energy was provided in liquid form (28). Soft drinks have become the leading source of sugar in the adolescent diet, contributing 36.2 g sugar/d for girls and 57.7 g sugar/d for boys (28). Other analyses of 1994 CSFII data for those aged 2–18 y (29) found that those subjects who consumed an average of 9 oz/d of caloric sodas had higher total EIs than did those who consumed none (2018 and 1830 kcal, respectively) and tended to consume less fruit juice and less fluid milk.

Numerous studies reported associations between soft drink consumption and body weight or body weight gain. Among these studies were those based on nationally representative cross-sectional surveys (8, 30), longitudinal cohorts (2, 5, 15, 31, 32), and between-group comparisons (33, 34). The data, as shown in Table 1, were mixed. Despite a dramatic rise in adolescent obesity rates, an analysis of NHANES III data showed that mean total EIs of youth aged 2–19 y changed little, except for a significant increase in adolescent females (8). In this age group, overweight youths consumed a significantly greater proportion of energy from beverages than did the nonoverweight youths (8). On the other hand, reanalyses of the nationally representative 1994–1996 CSFII dataset for 6–19-y-olds found that the consumption of caloric carbonated beverages, fruit drinks, and milk was unrelated to body weight (30). Instead, beverage choices and total beverage consumption were strongly linked to age, sex, and race (30).

Although cross-sectional studies can provide a fertile ground for speculation, they do not show causality. EIs, measured at a single point in time, can provide no indication of the direction of the association or of its possible relation to the dynamics of body weight. For example, epidemiologic studies generally show an inverse relation between the intake of sucrose (in any form) and the body weights of children (41–43) and adults (44, 45). That does not mean, however, that consuming more sugar will lead to weight loss. Given that sugar consumption declines with age, higher sugar intakes are usually associated with younger age, lower weight, and greater physical activity (46). Even when age is not a factor, cross-sectional studies may be confounded by activity patterns and energy needs, not to mention severe underreporting. In a study of 16 882 subjects aged 9–14 y, overweight participants reported consuming significantly less energy than did their normal-weight peers (47).

Between-group comparisons suggest that other dietary factors may also be involved. A study of 91 obese and 90 nonobese children and adolescents (aged 4–16 y) found that the obese group consumed significantly more sugar-sweetened drinks (excluding 100% fruit juice) than did the nonobese group (34). However, the obese group also consumed significantly more meat, grain products, potato chips, and total sugar, as determined by the dietary history method, whereas the consumption of many other sugar-rich foods (ie, cookies, candy, chocolate, doughnuts, and ice cream) did not differ significantly between the 2 groups. Another study, based on 14-d food records, showed no significant difference in soda consumption between 21 obese and 22 normal-weight adolescents (33). The normal-weight adolescents consumed twice the amount of high-calorie foods as did the obese adolescents (33). In a study of 928 men and 889 women aged 18–99 y in rural communities (36), overweight was associated with significantly more frequent consumption of soft drinks—but also with ordering super-sized portions, eating when watching TV, and not exercising enough.

A report based on the Nurses Health Study noted that the highest consumers of sugar-sweetened soft drinks were less physically active and were twice as likely to be current smokers than were the lowest consumers of soft drinks—21% compared with 10.9% (6). Physical activity is associated with socioeconomic status, which is in turn predictive of improved diet quality.
and better access to health care. Hence, it is difficult to establish links between obesity and the consumption of a single food, independent of economic variables that may also affect diet choice (48, 49).

BEVERAGES AND BODY WEIGHT CHANGE

Longitudinal cohort studies that address soft drink consumption and body weight change are extremely limited. Researchers have argued that longitudinal data, based on 2 points in time, permit better-informed speculation about causal links than do data from cross-sectional studies (15). Although such observations do provide evidence of parallel temporal trends, they still fall short of showing causality.

The most frequently cited study is one by Ludwig et al (15), which was based on a prospective 19-mo follow-up of 548 schoolchildren (7 ± SD age: 11.7 ± 0.8 y). Sugar-containing drinks were sodas, Hawaiian Punch (Dr. Pepper/Seven-Up, Plano, TX), lemonade, Kool-Aid (Kraft Foods, Northfield, IL), sweetened fruit drinks and iced tea, but not 100% fruit juice. Soft drink consumption at baseline was associated with BMI gains at follow-up (0.18/daily serving). For each additional daily serving during the study period, the children’s BMI increased by 0.24, after adjustment for anthropometric, demographic, dietary, and lifestyle variables. Whereas the consumption of sugar-sweetened drinks increased from 1.22 to 1.44 daily servings, fruit juice consumption declined from 1.28 to 1.08 daily servings, so that mean sugar consumption remained approximately the same. Despite the reported 37 new cases of obesity, the overall prevalence rate did not increase during the study period (27.4% compared with 27.7%). It can therefore be deduced that 35 children who were obese at baseline were no longer classified as obese at follow-up. Their beverage consumption was not mentioned (5).

Berkey et al (2) examined longitudinal changes in beverage consumption and BMI values in a large sample of 9–14-y-olds over 2 successive 1-y periods. Diets were assessed by using food-frequency questionnaires (FFQs), and BMI values were computed from self-reported heights and weights (2). At baseline, 23.2% of the boys and 17.5% of the girls were classified as overweight. In regression analyses, each additional daily serving of sugar-containing beverages (ie, soda, iced tea, and fruit drinks) was associated with a small gain (0.03–0.04) in BMI over 1 y. In other words, each additional 144 kcal of liquid sugar energy consumed per day (ie, 52 560 kcal/y) was associated with a body weight gain of only 100 g at the year’s end. Adjustment for total EIs further reduced the associations, which became nonsignificant. Whereas the data seemed to show that sugar-containing beverages had virtually no effect on body weight gain in growing children, that finding was at odds with the title of the study (2).

Welsh et al (5) studied 10 904 low-income children aged 2–3 y by using a retrospective cohort design. Dietary intakes at baseline were assessed by proxy report using FFQs. Heights and weights were measured at baseline and 1 y later. In normal-weight children, no association was found between soft drink

<table>
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<tr>
<th>Study (reference)</th>
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1 NA, not available; NHANES III, third National Health and Nutrition Examination Survey; CSFII, Continuing Survey of Food Intakes by Individuals; FFQ, food-frequency questionnaire.

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3 Completed by the child’s parent or guardian.

TABLE 1

Liquid sugar calories and overweight¹

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consumption at baseline and later weight gain. In contrast, children at risk (85th–95th percentile) and those already overweight (>95th percentile) who consumed >1 drink/d were twice as likely to become or remain overweight than were children in the referent group (<1 drink/d). That relation persisted after soda drinks were removed from the model. However, no dose–response relation was observed and, if anything, the highest consumption was associated with slightly lower risk.

Blum et al (31) examined the intakes of sugar-sweetened drinks, diet soda, 100% juice, and milk in 99 normal-weight and 48 overweight children (aged 9.5 y) over a period of 2 y. The consumption of sugar-sweetened drinks was not linked either to baseline BMI or to a change in BMI over the 2-y period, contrary to the previous report of Ludwig et al (15). Two recent studies using long follow-up periods (18–21 y) also failed to show the effect of sweetened beverages on body weight gain. One of these studies was based on data from the Bogalusa Health Study (38), and the other was based on a population of young adults in Norway (39).

Longitudinal data in adults are even more limited (6). One report, based on data from the Nurses Health Study, examined sharp changes in the consumption of different beverages in relation to body weight change over a 4-y period. Women who increased their beverage consumption from ≤1 serving/wk to ≥1 serving/wk were compared with the referent group of women who decreased consumption from ≥1 serving/d to ≤1 serving/wk, a major shift. For soft drinks, body weight gain was 4.7 kg versus 1.3 kg for the referent group. For 100% fruit juice, body weight gain was 4.0 kg versus 2.3 kg for the referent group, and for fruit punch the gain was 3.7 kg versus 2.4 kg. All 3 differences were significant (P <0.001). Greater consumption of soft drinks and fruit punch (but not of 100% juice) was also associated with a higher risk of diabetes (6).

A recent study examined the potential effect of soft drinks and fast foods on weight gain in a Spanish cohort of 7194 adult men and women (41 y) over a 28.5-mo period (32). Dietary intakes were based on a 136-item FFQ. Data analyses showed that soft drink consumption was linked to self-reported weight gain—but only in those participants who had gained 3–5 kg in the 5 y before the study. Soft drink consumption was unrelated to weight gain in persons whose prestudy weights were stable. In contrast, the consumption of fast foods (eg, hamburgers, pizza, and sausages) was associated with body weight gain, independent of weight history. These data suggest that the trajectory of body weight gain may be another important, and rarely measured, variable. Although soft drink consumption may exacerbate the problem in an already vulnerable population, other aspects of the diet may be important as well.

**DIETARY INTERVENTIONS**

Data on the success of interventions are equally sparse. One attempt to reduce soft drink consumption by 7–11-y-olds was based on a randomized, controlled study design (40). The educational program discouraged the consumption of caloric and diet carbonated soft drinks over the period of 1 school year in 6 primary schools. All children were encouraged to drink plain water. A 1-h educational session was assigned for each class each term, and additional support was available on the project’s website. The control group was not exposed to the program.

Consumption of carbonated soft drinks was measured in glasses per day (average glass size, 250 mL). At the end of 1 y, the intervention group consumed a smaller amount of caloric and diet carbonated soft drinks (0.6 glass/3 d), whereas the control group consumed slightly more (0.2 glass/3 d). (Because the reported drop in consumption included caloric and diet (0.3 glass for each) carbonated soft drinks, the decline in sugar energy can be estimated at 10 kcal/d. The drop in caloric soda consumption was not significant, and only the cumulative difference (caloric and diet sodas) reached P = 0.02. No significant group differences in BMI change were observed. The rates of overweight increased by 7.5% in the control group and decreased slightly (0.2%) in the intervention group. However, those differences cannot be attributed to a decline in the consumption of caloric sodas, because no significant decline was, in fact, observed.

A recent pilot study of 103 middle- to low-income adolescents aged 13–18 y tested the effect of replacing caloric beverages with diet beverages (50). Whereas the intervention group (n = 53) received free home delivery of diet beverages over a period of 25 wk, the control group (n = 50) continued their usual beverage consumption. Although EIs from the caloric beverages dropped by 82% (1201 kJ/d) in the intervention group, the difference in BMI gain was not significant (0.07 compared with 0.21). The effects of the intervention on BMI were significant only for those 18 adolescents (12 intervention and 6 control) whose baseline BMIs were >30. Adolescents in the top tertile of BMIs who were in the intervention group showed a modest weight loss (BMI decrease of 0.63 or weight loss of ≈1.75 kg), whereas those in the control group continued to gain weight (BMI increase of 0.12). In this study, diet beverages had the greatest effect on body weight in the group that needed them the most. The study was also one of the few that took economic factors into account (50).

**CONTEXT AND BEHAVIORAL INTENT**

Observational studies, whether cross-sectional or longitudinal, provide no information as to why people consume a given food or beverage. This point can be illustrated with reference to the consumption of zero-calorie diet soft drinks, for which the data are highly inconsistent. For example, at baseline, Ludwig et al (15) found no association between diet soda consumption and BMI. In contrast, Berkey et al (2) found a positive association at baseline, but only in boys. Analyses of the cross-sectional 1994–1996 CSFII dataset for 2–19-y-olds found a weakly positive association between diet soda consumption and BMI (30): it was the overweight youth who consumed more diet sodas.

The effect of diet soda consumption on body weight change was not clear. Whereas one study found that diet soda consumption was associated with lower obesity risk (15), another study found that diet soda consumption was associated with higher obesity risk (2). In the second study, the association between diet cola consumption and higher obesity risk was significant in boys but not in girls. In other words, the same studies that linked the consumption of caloric sodas to weight gain (2, 15) linked diet soda consumption to weight gain (2) or to weight loss (15). The authors attributed this discrepancy to the heavier participants’ presumed intent to lose weight (2). However, in the absence of information on dieting practices, behavioral intent should not be imputed post hoc from purely observational data. On the basis of
the observed association between increased soft drink consumption and increased EI from other foods. Schulze et al (6) suggested post hoc that the consumption of caloric beverages may have induced hunger and thus food intake. An alternative hypothesis—that the increased consumption of solid foods induced thirst—was not examined.

LABORATORY STUDIES OF SUGAR AND WEIGHT GAIN

Experimental evidence linking daily soft drink consumption with increased EI and body weight gain is provided by 3 studies (Table 2). Two were conducted with normal-weight adults who were asked to consume large volumes of sugar-sweetened beverages in addition to their usual diet (51, 52). The third was conducted with overweight adults, who were provided with sucrose- or sweetener-containing beverages and foods in addition to their usual diets for a period of 10 wk (53).

One study provided normal-weight participants with 1135 g of beverages sweetened with either HFCS (530 kcal or 2215 kJ) or aspartame (51). The consumption of caloric and diet sodas reduced the intake of energy from the diet by a nonsignificantly different amount (179 and 195 kcal/d, respectively), which suggests only a partial compensation. As a result, total EI (including sodas) were higher in the HFCS condition, leading to body weight gain.

Raben et al (53) provided overweight adults with sodas and juices and with solid foods (ie, yogurt, marmalade, ice cream, and stewed fruits) containing either sucrose or intense sweeteners, mostly aspartame. Minimum mandatory consumption of sucrose was set at 2 g·kg⁻¹·d⁻¹. Persons in the 60–75 kg weight range consumed 125 g sucrose/d, those in the 75–90 kg weight range consumed 150 g/d, and those weighing >90 kg consumed 170 g/d. The sucrose condition provided an average of 28% of energy from sucrose, or ≈152 g/d, 70% of which came from beverages. The energy density of the diet was higher in the sucrose than in the sweetener condition.

Mandatory consumption of sucrose in addition to the usual diet led to higher EI and to body weight gain. After 10 wk, the sucrose group had higher EI (1.6 MJ/d), body weight (1.6 kg), fat body mass (1.3 kg), and blood pressure, whereas no change or a decrease was observed in the sweetener group. The likely reason for these differences, according to the authors, was that the provision of sucrose in largely liquid form failed to promote satiety (53).

Using a crossover study design, Van Wyk et al (52) asked young normal-weight subjects to consume 2 L/d (2000 mL or 68 oz) of a distinctively flavored sucrose-sweetened or sugar-free beverage. Food consumption was measured over 2 d. No downward adjustment in diet energy was observed, so that total EI over 2 d were higher in the sucrose than in the sweetener condition. The participants were then habituated for 4 wk to the distinctive tastes of the caloric and noncaloric drinks, in an effort to promote associative learning. Nonetheless, at the end of 4 wk, they still ingested more energy in the sucrose than in the sweetener condition. In all 3 studies (51–53), mandatory consumption of sugar-sweetened beverages in addition to the usual diet led to higher EI, a situation that was not corrected after habituation and learning.

CLINICAL STUDIES OF LIQUID SUGAR AND WEIGHT LOSS

Liquid MR products used in clinical studies were sugar solutions supplemented with small amounts of protein and fiber. A typical 325-mL Slim Fast liquid MR shake (Unilever, Rotterdam, Netherlands) contained 36 g sugar (144 kcal out of a total of 220 kcal), 10 g protein, and <2 g fat. The amount of liquid sugar calories was comparable to that in 12-oz caloric soft drinks (34 g/12 oz). In some product formulations, HFCS was listed as the principal sweetener. Although the sugar content of most MR shakes has since been reduced, virtually all studies published in peer-review literature were based on the full-sugar version (26).

The success of HFCS-sweetened liquid sugar shakes in promoting weight loss has been documented in several clinical trials (26). Some of the published studies were based on randomized controlled clinical trials, the gold standard of evidence-based medicine. The partial MR (PMR) program was a low-calorie diet (800–1600 kcal/d) in which up to 2 meals were replaced with liquid MR shakes or with solid bars. The shakes typically provided 30–72 g/d of liquid sugar energy. One 220-kcal solid MR bar contained 20–24 g sugar (average: 22 g), 8 g protein, and 5 g fat. Other products contained ≈20 g of sugar, 1–3 g fat, 7–10 g protein, and up to 5 g fiber (26).

Uniformly described as palatable, inexpensive, and convenient (26, 54), sweetened liquid shakes provided a daily sugar dose (30–72 g/d) that often exceeded the one now associated with soft drinks (30–60 g/d). In one study (55), the MR group was instructed to replace 3 meals/d with a 220-kcal liquid shake, for a total of 108 g sugar/d. In another study (56, 57), 2 of 3 main meals were replaced with liquid shakes, soups, or hot chocolate. Each liquid shake contained 0.84–1.05 MJ, including 27–33.5 g carbohydrate, most of which was sugar. In addition, 2 daily snacks were replaced with solid snack replacement bars that contained 0.38–0.46 MJ energy and 16.1–18.1 g carbohydrate, mostly sugar. HFCS was a key ingredient. Daily sugar consumption, both as liquid and solid, was therefore on the order of 90 g. Ashley et al (58, 59) instructed participants to replace 2 of 3 main meals with liquid shakes containing 35–41 g carbohydrate or with solid bars containing 31–34 g carbohydrate for a total of ≈70 g added sugar/d. The proportion of energy from sugars was ≈25–33%, which is comparable to the 28% reported by Raben et al (53), and most of the sugar energy was provided in liquid form. The efficacy of these sugared liquids in promoting weight loss was then compared with that of conventional reduced-calorie diets.

Heymsfield et al (26) conducted a useful meta-analysis and pooling analysis of randomized controlled PMR interventions lasting ≥12 wk in subjects aged >18 y and with a BMI > 25. Studies with self-reported weights and heights were excluded. Of 30 studies conducted between 1960 and 2001, only 6 met all the inclusion criteria in using liquid MR products and a control condition with an associated diet plan (see Table 2).

All analyses pointed to a significantly greater weight loss in subjects who consumed liquid sugar energy than in those following isocaloric conventional diets. At 3 mo, 72% of the PMR group lost >5% of body weight, whereas only 34% of subjects on the conventional diet did so. Mean weight loss in the PMR group was 2.5 kg greater at 12 wk and 2.4 kg greater at 12 mo than that in the conventional diet group. Pooling analysis
### Table 2

Laboratory studies and randomized controlled clinical trials of the effects of sugar solutions on weight change

<table>
<thead>
<tr>
<th>Study (reference)</th>
<th>Subjects</th>
<th>Age</th>
<th>Intervention</th>
<th>Active phase</th>
<th>Estimated sugar intake</th>
<th>Paid for by subjects?</th>
<th>Control group intake</th>
<th>Paid for by subjects?</th>
<th>Contact</th>
<th>Weight change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Laboratory</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tordoff and Alleva (51)</td>
<td>30</td>
<td>22–30</td>
<td>2.2 MJ/d</td>
<td>3 wk</td>
<td>133 g/d</td>
<td>Yes</td>
<td>Aspartame; no beverage</td>
<td>Yes</td>
<td>10 Visits</td>
<td>Gain with HFCS</td>
</tr>
<tr>
<td>Raben et al (53)</td>
<td>41</td>
<td>20–50</td>
<td>3.4 MJ/d</td>
<td>10 wk</td>
<td>152 g/d</td>
<td>Yes</td>
<td>Intense sweeteners</td>
<td>Yes</td>
<td>11 Visits</td>
<td>Gain (1.3–1.6 kg)</td>
</tr>
<tr>
<td><strong>Clinical</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ditschuneit et al (56)</td>
<td>100</td>
<td>18–65</td>
<td>2 MR, 2 SR</td>
<td>12 wk</td>
<td>102 g/d</td>
<td>Yes</td>
<td>5.0–6.2 MJ/d</td>
<td>No</td>
<td>12 Visits</td>
<td>Loss (7.1 versus 1.3 kg)</td>
</tr>
<tr>
<td>Rothacker et al (55)</td>
<td>75</td>
<td>18–55</td>
<td>3 MR (5.0 MJ/d)</td>
<td>12 wk</td>
<td>108 g/d</td>
<td>Yes</td>
<td>5.0 MJ/d</td>
<td>No²</td>
<td>None</td>
<td>Loss (6.3 versus 3.8 kg)</td>
</tr>
<tr>
<td>Yip et al (60)</td>
<td>57</td>
<td>&gt;30</td>
<td>2 MR (2.0 MJ/d)</td>
<td>12 wk</td>
<td>11–72 g/d</td>
<td>NA</td>
<td>ADA diet: 2.0 MJ/d</td>
<td>No</td>
<td>5 Visits</td>
<td>Loss (6.1 versus 4.2 kg)</td>
</tr>
<tr>
<td>Ashley et al (58, 59)</td>
<td>113</td>
<td>25–50</td>
<td>2 MR/d (shake or bar)</td>
<td>12 mo</td>
<td>72 g/d</td>
<td>Yes</td>
<td>Lifestyle group</td>
<td>No</td>
<td>26 Visits</td>
<td>Loss (3.5–7.7 versus 3.4 kg)</td>
</tr>
<tr>
<td>Allison et al (61)</td>
<td>74</td>
<td>35–65</td>
<td>MR (5.0 MJ/d)</td>
<td>12 wk</td>
<td>75 g Carbohydrate/d</td>
<td>NA</td>
<td>1200 kcal/d</td>
<td>NA</td>
<td>1 Visit</td>
<td>Loss (7.0 vs 2.9 kg)</td>
</tr>
<tr>
<td>Noakes et al (62)</td>
<td>66</td>
<td>20–65</td>
<td>2 MR (6.0 MJ/d)</td>
<td>12 wk</td>
<td>72 g/d</td>
<td>Yes</td>
<td>Structured meal</td>
<td>Yes⁴</td>
<td>6 Visits</td>
<td>Loss (6.6 kg); NS</td>
</tr>
<tr>
<td>Poston et al (63)</td>
<td>100</td>
<td>33–55</td>
<td>MR (5.0–6.2 MJ/d)</td>
<td>24 wk</td>
<td>MR</td>
<td>NA</td>
<td>MR and snacks</td>
<td>Yes</td>
<td>NA</td>
<td>Loss in all conditions</td>
</tr>
<tr>
<td>Ebbeling et al (50)</td>
<td>103</td>
<td>13–18</td>
<td>Diet beverages</td>
<td>25 wk</td>
<td>Decrease of 1.2 MJ/d</td>
<td>Yes</td>
<td>Regular beverages</td>
<td>No</td>
<td>6 Telephone calls</td>
<td>Loss only for subjects with BMI &gt;30</td>
</tr>
</tbody>
</table>

¹ HFCS, high-fructose corn syrup; MR, meal replacement; SR, snack replacement; NA, not available; ADA, American Diabetes Association.
² $400 Honorarium for each participant.
³ Payment of $50 Australian/2 wk.
strengthened these effects. Overall, the magnitude of the weight-loss effect induced by drinking liquid sugar shakes (ie, 36–72 g sugar/d) was said to be in the range observed in pharmacologic studies (26).

These clinical data stand in stark contrast to the prevailing notion that the consumption of liquid sugar energy has inevitable metabolic consequences leading to weight gain (1, 4, 17, 20). Rather, the studies indicate that sugared MR shakes can safely and effectively produce a significant weight loss (26). The shakes were undoubtedly liquid, they supplied significant sugar energy, and, in some cases, they contained HFCS.

Later studies confirmed the efficacy of liquids in promoting weight loss. Using soy-based liquid shakes with a higher protein and lower sugar content (13 g), Allison et al (61) found that the PMR group lost significantly more weight than did the control conventional diet group (7.0 and 2.9 kg, respectively). In a recent study (63), 100 participants were randomly assigned to 1200–1500 kcal/d diets and asked to consume 2 MR products/d with or without snacks. By 24 wk, mean weight loss across the 2 groups was 4.6 kg. The literature provides further examples of liquid weight-loss diets. In one study, a weight-reducing diet composed only of milk (5.6 MJ/d) led to a loss of 9.4 kg over 16 wk (64). None of the studies reported significant problems with satiety that were specifically due to the consumption of liquid foods. Indeed, liquid sugar shakes were said to be more effective in promoting weight loss than were diets high in vegetables and fruit (26).

The high sugar content of MR shakes used to treat obese diabetic patients has been a concern for some investigators. Yip et al (60) compared the effects of canned liquid shakes containing lactose (11 g), fructose (13 g), and sucrose (8.5 g) on weight loss with the effects of sugar-free shakes containing equivalent concentrations of maltodextrins. Weight losses in the 2 PMR groups were similar and significantly higher than those in the sparkling water control condition. Virtually identical intervention was kept to a minimum (55, 66). One 12-wk study showed that unsupervised patients provided with liquid shakes outside the clinical setting still managed to lose weight (65). The consumption of liquid MR products, whether unsupervised (62) or in a pharmacy setting (67), was just as effective in weight management as the conventional reduced-calorie diets (62, 67).

One study was based on the provision of MR products, free of charge, to 141 overweight participants in rural Wisconsin for a period of 5 y (66). At the end of the study, the sample of 50 men lost a mean of 5.8 ± 5.4 kg, and the 84 women lost 4.2 ± 6.9 kg. In contrast, 142 men in the control group (selected post hoc) gained 6.7 ± 10.2 kg, and 247 women gained 6.5 ± 10.7 kg (66).

The literature repeatedly noted that the shakes were palatable, convenient, and readily accepted by the patients (26). No mention was made of hunger or satiety deficits, and no suggestion was made that liquid calories were not perceived by the body. On the contrary, participants in the PMR group reported a high level of hunger satisfaction (65).

LIQUID CALORIES AND SATIETY

The notion that liquid calories fail to trigger satiety mechanisms (17, 22) is routinely cited to support associations found in epidemiologic studies (1, 4, 6). Yet, a review of the satiety literature found much of it to be inconclusive (23). For the most part, studies of beverages and satiety have measured the short-term effect of a caloric preload, ingested in the laboratory, on hunger and satiety ratings and on EI at the next meal. Those mechanisms may not translate to dietary patterns measured in the long term. Furthermore, in many of those studies, energy adjustments after ingestion of a preload were influenced by subject characteristics, preload volume, and nutrient composition and by the interval between the preload and the test meal. These factors were often more important than whether the preload was liquid or solid (23).

Despite claims that all liquid calories are not perceived by the body (17), nutritionists have encouraged replacing sodas with 100% fruit juices and with low-fat milk (68). One important question, therefore, is whether the satiating power of soft drinks is the same as that of 100% fruit juices and milk (23, 69, 70). The latter 2 beverages were described as “foods that you drink,” which are capable of triggering physiologic satiety (69, 70). Although their sugar content does vary, caloric cola, orange juice, and milk (1% fat) have equivalent energy density—≈0.4 kcal/g. In one study, the 3 beverages (250 kcal) had identical effects on the temporal profiles of hunger, fullness, and desire to eat for up to 2 h after ingestion (69). However, no energy adjustment at lunch was observed, so that total EI was higher than those in the sparkling water control condition. Virtually identical results were obtained when 150 kcal caloric cola, orange juice, and 1% milk was consumed with a meal and before a meal (71).

Although both studies showed that caloric beverages had little effect on the next meal, there were no major differences between sugared cola, 100% fruit juice, and low-fat (1%) milk (23). Another study, also based on the preload paradigm, failed to show significant differences in satiety profiles of a cola beverage and...
an isocaloric amount of solid cookies (24). Both suppressed intake when consumed immediately before lunch (24).

Arguably, the effect of milk or juices on satiety may be modulated by the protein or fiber content of these beverages. MR shakes typically contain protein (13.5 g) and some fiber (5 g), both of which are known to have an effect on satiety, whereas soft drinks generally contain neither. MR shakes and 1% milk have similar protein content (2.9 g/100 g for an MR shake and 3.0 g/100 g for milk). MR shakes contain 5 g fiber/350 mL (1.4 g/100 g), whereas milk and orange juice contain none.

The evidence for the satiating power of protein when administered in beverage form is extremely limited. Although protein is reputed to be more satiating than either sugar or fat, studies have failed to show a strong satiety effect of 1% fat milk (23, 71). On the other hand, drinkable yogurt containing 17.1 g protein/378 g was more satiating than a dairy fruit drink (2.6 g protein/400 mL) or a fruit drink (0 g protein), that were matched for both volume and energy content (72). It may be that protein’s satiating effects are threshold dependent or related to protein type, or that they are simply too elusive for the conventional preload study design. Anderson et al (73) found that participants who consumed liquid preloads of milk-based protein (whey) ate more at the next meal than those who consumed liquid preloads of egg-albumen protein.

Dietary fiber has also been associated with greater satiety (74). However, most studies on fiber and satiety have been conducted not with beverages but with solid or semi-solid foods (75, 76), although some studies were conducted with beverages (77). In one study, fiber (< 0.1 g microcrystalline cellulose) added to a beverage increased viscosity and led to higher satiety ratings (78). Participants reported significantly greater satiety when fiber was present in a fruit puree or was added to the stimulus as soluble plant fibers than when it was not present (79). The physiologic mechanisms proposed for the satiating effects of fiber include slowed ingestion time because of the need for chewing, increased gastric distention (signaling fullness), and delayed gastric emptying (74) As yet, no data clarify whether the supposed satiety deficit after the ingestion of sugar-containing liquids can be counteracted by the ingestion of fiber.

The supposed contribution of fructose to weight gain is largely based on extrapolations from animal studies (22). Whereas, in many such studies, the animals were fed pure fructose, HFCS used in soft drinks contains 55% fructose and 45% glucose. Differences in fructose metabolism are minimized when small amounts of glucose are present (80). Almost no studies on fructose and weight gain in humans have been published (21).

THE COST OF LIQUID SUGAR

MR shakes were consistently described as a less costly alternative to very-low-calorie diets (VLCDs) and to prepared foods (62). The point has frequently been made that such shakes cost less than the meal they replace (81). Furthermore, in most clinical studies, shakes and snack bars were provided to the dieting patients at no cost. Ashley et al (58, 59) provided coupons to be redeemed at local stores and distributed snack bars at scheduled group sessions. Winick et al (65) used weekly delivery of 14 powder shakes and 12 snack bars to participants who needed only to buy skim milk. Rothacker (66) provided free MR products for 5 y to 141 overweight adults in rural Wisconsin. In contrast, the instructions to follow a low-fat diet with plenty of vegetables and fruit were not accompanied by any financial incentive. A few studies either paid all participants $25/wk over 12 wk (54) or provided $600 stipends and some free groceries to all participants (82).

Ebbeling et al (50) also provided an economic incentive by supplying sugar-free products to a sample of 103 nondieting adolescents, one-third of whom were obese. Bottled water and sugar-free diet beverages were delivered to each subject’s home over a 25-wk period. Each household received the equivalent of four 12-oz servings of noncaloric beverages per day for the study participant and 2 servings/d for each additional household member. A supermarket delivery service filled the orders, delivering 3–5 times/wk. Participants were contacted monthly by telephone to discuss satisfaction and provide motivational counseling, but no other professional intervention was provided. At the end of the study, all participants, including the control group, received a $100 gift certificate for use at a local shopping mall (50).

Although monetary incentives are known to improve dietary compliance (83, 84), few studies have explored the effect of providing free food to study participants. Providing foods with up to 660 kcal/d free of charge could not have been an insignificant factor for subjects whose reported mean annual income was $10 420 (66). Receiving beverages worth $1.50–$2.00 a day (50), whether caloric or not, may have represented a substantial saving in the food budget of minority families living in subsidized housing in Boston. The population in that study was 66% nonwhite, and 40% had a household income <$30 000 (50). It is interesting that the reported outcome was weight loss, regardless of whether the supplied beverages were sugared (26) or sugar-free (50).

It may be that the influence of liquid sugar calories on weight control involves economics more than it does human physiology. According to Keogh and Clifton (27), studies that showed the superiority of liquid shakes over conventional diets were those in which the MR shakes were provided for free, but subjects had to pay for the conventional diets. Only one study provided control participants with shopping vouchers with a financial value similar to that of the MR products (62). When equivalent financial incentives were provided to controls, the 2 conditions no longer differed significantly.

Obesity in the United States is associated with limited resources (48). Sugar-sweetened beverages are an inexpensive source of energy in the typical US diet (28). Soft drinks, 100% fruit juices, and MR shakes have roughly the same sugar content per 100 g, as shown in Table 3. The sugar content of colas, soft drinks, fruit punches, 100% fruit juices, and liquid shakes is ≈10–12 g/100 g, which is close to the hedonic optimum for sweet taste. Although some of the currently available liquid shakes have reduced their sugar content by 50%, higher-sugar versions that provide as much sugar as do soft drinks are still available. On the other hand, soft drinks, juices, and MR shakes differ sharply in their energy cost ($/MJ). Whereas soft drinks cost ≈$2.50/MJ, the cost per MJ of 100% fruit juices was several times higher. At the high end of the scale, liquid MR shakes cost up to 10 times as much per MJ. Even though the sugar content of all beverages was approximately the same, it was the lower-cost beverages that have been most consistently associated with weight gain (1, 3, 4).
LIQUID CALORIES, SUGAR, AND BODY WEIGHT

The argument that liquid calories are not detected by the body (17) has been used to establish a causal connection between the consumption of sweetened beverages and body weight gain. For the most part, the debate about soft drinks and overweight has been couched in biomedical terms, and much attention has been paid to short-term satiety deficits, energy compensation, and sugar metabolism (1, 4, 6, 20, 21). Even though short-term satiety and the long-term regulation of body weight are distinct mechanisms, beverages are said to contribute to obesity by virtue of being liquid and having a high sugar content (4, 6).

However, regular daily consumption of sugared liquids need not automatically result in weight gain. Clinical evidence has shown that regular consumption of sugar-containing liquid MR products by overweight patients can lead to a significant and sustained weight loss (26). Liquid MR shakes, provided free of charge, are the preferred treatment modality in the Look AHEAD (Action for Health in Diabetes) Study (85), a multicenter, randomized controlled trial of 5145 participants undergoing intentional weight loss. Liquid MRs were included in the 1200–1500 kcal diet because they simplified food choices (85), improved glycemic control (85), and led to a significantly greater weight loss as compared with isocaloric diets composed of conventional foods (85).

Given that the consumption of sugared liquids can be linked to loss as well as to weight gain, it may be time to focus on dietary behavior (86). Whereas sugar-sweetened soft drinks are consumed with meals, sugar-sweetened MR shakes are consumed instead of meals (26, 85). Evidently, the critical issue is not sugar metabolism but the way that sugar is used by the consumer. In a departure from the notion that all liquids fail to promote satiety (17, 22), one brand of canned liquid MR shakes is marketed with such slogans as “helps control hunger” and “prevents hunger longer” (87), which presumably had been approved by the Federal Trade Commission.

The current advice would be to focus on the psychological as well as the physiologic aspects of weight management. Successful weight management requires cognitive control of EIIs, a healthy lifestyle, and successful adherence to low-energy diets (85). And if context and dietary behavior become the key issues, then the notion of a physiologic satiety deficit after liquid sugar consumption (17) loses much of its popular appeal.

At this time, the epidemiologic evidence linking beverage consumption to the global obesity epidemic is still weak (88). Given that most of the studies were conducted in the United States (1, 3, 8), a clear need exists for additional international comparisons. For example, although France has experienced a sharp rise in childhood obesity rates (89), the consumption of sugars has traditionally been low (10–15 g/d), HFCS has not entered the food supply in significant amounts, and the consumption of soft drinks is far lower than in other European countries (89). Because of a trade dispute, Mexico instituted in 2003 an internal tax on US-manufacturered HFCS, which resulted almost immediately in the near-total replacement of HFCS in soft drinks with sucrose (90). Whether this measure will reduce the high prevalence of overweight and obesity in Mexican children (91) remains to be seen.

The cost of sweetened beverages, documented in Table 3, is another unexplored issue. It would appear that the obesity-promoting capacity of different beverages is linked not so much to their sugar content (which is the same) but to their low price. Obesity has been linked to limited economic resources (48) and may involve preferential selection of low-cost beverages and foods (49). Studies of diet sugar content, dietary choices, and health outcomes should take diet costs into account (48, 49).

Both authors participated equally in the literature search and review and in all phases of writing and revising the manuscript. Neither author had a personal or financial conflict of interest.

REFERENCES


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**TABLE 3**
The price of sweetened beverages

<table>
<thead>
<tr>
<th>Beverages</th>
<th>Sugar content</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/100 g</td>
<td>$/MJ</td>
</tr>
<tr>
<td>Tampico Tropical Punch</td>
<td>10.8</td>
<td>0.25</td>
</tr>
<tr>
<td>Safeway Select Cola</td>
<td>12.1</td>
<td>0.32</td>
</tr>
<tr>
<td>Reduced-fat Chocolate Milk</td>
<td>12.3</td>
<td>0.38</td>
</tr>
<tr>
<td>A&amp;W Root Beer</td>
<td>12.9</td>
<td>0.40</td>
</tr>
<tr>
<td>Coca-Cola Classic</td>
<td>11.3</td>
<td>0.44</td>
</tr>
<tr>
<td>Sunny D Tangy Original (5% juice)</td>
<td>11.3</td>
<td>0.44</td>
</tr>
<tr>
<td>Hi-C Blast Fruit Pow (10% juice)</td>
<td>12.7</td>
<td>0.66</td>
</tr>
<tr>
<td>Safeway White Grape Juice</td>
<td>15.8</td>
<td>0.77</td>
</tr>
<tr>
<td>(100% juice)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Welch’s Grape Juice (100% juice)</td>
<td>16.7</td>
<td>0.82</td>
</tr>
<tr>
<td>Ocean Spray Cranberry Juice Cocktail (27% juice)</td>
<td>13.8</td>
<td>0.89</td>
</tr>
<tr>
<td>Minute Maid Orange Juice (100% from concentrate)</td>
<td>10.0</td>
<td>0.94</td>
</tr>
<tr>
<td>Tropicana Orange Juice (100% pure squeezed)</td>
<td>9.2</td>
<td>1.08</td>
</tr>
<tr>
<td>V8 Fusion Fruit &amp; Vegetable Juice (100% pure from concentrate)</td>
<td>11.2</td>
<td>1.49</td>
</tr>
<tr>
<td>Slim Fast Optima French Vanilla (low sugar)</td>
<td>4.8</td>
<td>1.59</td>
</tr>
<tr>
<td>Slim Fast French Vanilla Classic (original formula)</td>
<td>10.8</td>
<td>1.59</td>
</tr>
<tr>
<td>Odwalla Orange Juice (100% juice squeezed)</td>
<td>10.0</td>
<td>2.92</td>
</tr>
</tbody>
</table>

---

1 All prices were obtained from www.safeway.com (accessed 23 March 2006).
2 Heartland Farms, City of Industry, CA.
3 Safeway Inc, Pleasanton, CA.
4 Darigold Dairies, Seattle, WA.
5 Dr. Pepper/SevenUp Inc, Plano, TX.
6 Coca-Cola Company, Atlanta, GA.
7 Sunny Delight Beverages, Cincinnati, OH.
8 Welch Foods Inc, Concord, MA.
9 Ocean Spray Cranberries Inc, Lakeville-Middleboro, MA.
10 PepsiCo Inc, Purchase, NY.
11 Campbell’s, Camden, NJ.
12 Unilever, Vlaardingen, Netherlands.


Skeletal muscle lipid deposition and insulin resistance: effect of dietary fatty acids and exercise¹⁻³

Michael P Corcoran, Stefania Lamon-Fava, and Roger A Fielding

ABSTRACT
Mounting evidence indicates that elevated intramyocellular triacylglycerol concentrations are associated with diminished insulin sensitivity in skeletal muscle. This lipid accumulation is most likely due to enhanced fatty acid uptake into the muscle coupled with diminished mitochondrial lipid oxidation. The excess fatty acids are esterified and either stored or metabolized to various molecules that may participate or interfere with normal cellular signaling, particularly insulin-mediated signal transduction, thus altering cellular and, subsequently, whole-body glucose metabolism. Impaired insulin responsiveness, if not managed, can further progress to type 2 diabetes mellitus, an all too common condition. For most of the human population this is avoidable, given that causes of intramyocellular lipid deposition are predominantly lifestyle-mediated. Chronic overconsumption of calories coupled with deleterious intakes of saturated or trans-unsaturated fatty acids inconsistent with the recommendations outlined in the Dietary Guidelines for Americans have been shown to increase the risk of insulin resistance. Furthermore, lack of exercise, which can have a profound effect on skeletal muscle lipid turnover, is implicated in this lipid-induced insulin resistance. This review summarizes the current understanding of the effects of elevated intramyocellular lipids on insulin signaling and how these effects may be altered by varying dietary fat composition and exercise. Am J Clin Nutr 2007;85:662–77.

KEY WORDS Insulin resistance, skeletal muscle, intramyocellular triacylglycerol, dietary fat, exercise

INTRODUCTION
With the substantial increase in the rate of obesity in both children and adults during the past few decades, concern has risen over the increasing prevalence of morbidity and mortality affiliated with this condition. The incidence of obesity is currently of epidemic proportion, and there are no signs that it will decrease, given the current trend. Obesity, a condition characterized by excess body fat, is defined as a body mass index (in kg/m²) ≥ 30 (a body mass index of 25–29 indicates overweight status). Currently, 30% of American adults are classified as obese and 1 of every 6 children are overweight according to the National Health and Nutrition Examination Survey (1). The likelihood of becoming obese does not depend on sex, age, or ethnicity, yet disparities do exist in their prevalence, and children who are overweight have an increased likelihood of becoming obese adults (2, 3). This increase in the incidence of obesity is undoubtedly an important contributor to the increase in insulin resistance (4) and the metabolic syndrome (5), as well as in type 2 diabetes mellitus (T2DM) among both children and adults (4).

Despite the fact that the epidemiologic correlations are well established, the pathophysiology of obesity, particularly with regard to insulin resistance, has yet to be clearly defined. Insulin resistance, a fundamental feature of T2DM, is characterized as the tissues’ inability to take up glucose in response to the pancreatic hormone insulin. Skeletal muscle has been identified as the major tissue in glucose metabolism, accounting for ≈75% of whole-body insulin-stimulated glucose uptake (6, 7), and insulin resistance has been associated with accumulation of body fat (8), particularly intramyocellularly in both animals (9, 10) and humans (11, 12). This suggests a possible causative role for skeletal muscle lipid oversupply associated with chronic obesity in the development of insulin resistance (13). However, this assumption has not always been validated, because studies have also shown 1) improvements in skeletal muscle insulin sensitivity with little to no change in intramyocellular lipid concentrations (14, 15) and 2) improvements in skeletal muscle insulin sensitivity coinciding with actual increases in intramyocellular lipid concentrations (16). Furthermore, elite endurance athletes have extremely high concentrations of muscle lipid, yet are also quite insulin sensitive (17). The nature of this metabolic paradox seems to indicate that it is not the size of the intramyocellular triacylglycerol (IMTG) pool, but rather the balance between fatty acid availability, cellular uptake, and oxidation (ie, lipid turnover). Thus, the cellular and molecular mechanisms linking obesity to lipid-induced insulin resistance are currently a topic of intense investigation, and prevailing theories speculate that lipotoxic effects are mainly due to metabolites derived from intramyocellular lipid metabolism in addition to alterations in membrane function through changes in sarcolemma fluidity (13, 18, 19).

To further elucidate how elevated plasma and intramyocellular free fatty acids (FFAs) affect insulin signaling on a mechanistic level and how these defects can potentially be normalized

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by lifestyle modifications, a detailed understanding of the extent to which increased muscle lipids act in the insulin signaling pathway needs to be determined. In the present review, current knowledge on the implications of intramyocellular lipid deposition on skeletal muscle metabolism, particularly in regard to insulin resistance, is presented. First, mechanisms of lipid accumulation and fat distribution in muscle will be discussed, followed by a general overview of the alterations in insulin signaling that have been shown to occur, including possible lipid mediators linked to insulin resistance. Finally, the effect of exercise and of manipulating dietary fat composition will be addressed.

MECHANISMS OF INTRAMYOCYTOPLASMIC LIPID DEPOSITION

Skeletal muscle lipid metabolism

Plasma lipid concentrations play a role in determining the rate of uptake of FFA into the muscle, particularly during conditions of hyperinsulinemia (20), which is often present with insulin resistance. Interestingly, circulating FFA concentrations are usually elevated in obese persons. Fasting plasma FFA concentrations in obese and T2DM patients typically range from 600 to 800 μmol/L compared with 300–400 μmol/L in lean healthy persons (21). This, coupled with reduced lipid oxidation often exhibited in obese skeletal muscle (22), results in excessive intramyocellular lipid deposition. Consequently, the excess muscle FFAs are either stored in lipid droplets or converted to various signaling molecules. This FFA conversion “spill over” is predominately due to increased availability of fatty-acyl-CoA substrates for enzymes involved in synthesis of sphingolipids, eicosanoids, phospholipids, etc, and results in abnormal concentrations of these respective molecules, which may play a significant role in lipid-mediated insulin desensitization.

In order for lipids to be used as fuel by skeletal muscle, FFAs must be taken up and converted intracellularly to long-chain fatty acyl-CoAs (LCACoAs), imported into the mitochondria by carnitine acyltransferases, and subjected to β-oxidation. However, LCACoAs also serve as a source of second messengers, such as diacylglycerol (DAG) or ceramide, through de novo synthesis or through phospholipase C activation or phospholipid hydrolysis, as shown in Figure 1. **FIGURE 1.** Metabolism of free fatty acids to long-chain fatty acyl-CoAs (LCACoAs). LCACoAs can either be used for energy production through β-oxidation or undergo conversion to various signaling molecules, such as ceramide and diacylglycerol (DAG). Because obese persons have higher concentrations of intramyocellular fatty acids than do lean persons, the abundant supply of LCACoAs in skeletal muscle is favored toward signal molecule production. FATP, fatty acid transport protein; HS-CoA, coenzyme A.

**Lipid transport and tissue delivery**

Fatty acids are transported in the blood bound to albumin as nonesterified fatty acids or as part of triacylglycerols in lipoprotein complexes, which typically require triacylglycerol hydrolysis via lipoprotein lipase (LPL) to deliver the fatty acids to the tissues. Overexpression of muscle LPL has been associated with insulin resistance (23, 24), possibly because of the effect LPL has on increasing intramyocellular triacylglycerol concentrations (23, 25), though this effect has not always been consistent. For example, Voshol et al (25) showed no effect on insulin-stimulated whole-body or muscle-specific glucose uptake using mice overexpressing LPL. This argues against a simple causal relation between intramyocellular triacylglycerol content and insulin resistance. The discrepancy may be explained by differences in experimental conditions, such as the genetic background of the mouse models used, dietary fat content, body weight, muscle and liver triacylglycerol content, and insulin concentrations during the hyperinsulinemic-euglycemic clamp. Regardless, the authors did show that elevated LPL caused alterations in intracellular glucose metabolism, including decreased glycolysis, glucose oxidation, and glycogen synthesis (25), indicating possible abnormalities downstream of...
insulin interaction with its receptor independent of GLUT4 trafficking (see “Insulin-mediated signaling transduction and glucose uptake” section).

**Cell-mediated lipid uptake**

Fatty acid transport proteins (FATPs) are implicated in the facilitated cellular uptake of lipids and their activation via ligation to acetyl-CoA. Lipid uptake by muscle tissue occurs mainly via the fatty acid transport protein 1 (FATP-1), a 640 amino acid integral plasma membrane protein that is expressed in all cells requiring high levels of fatty acid uptake for storage or metabolism (26–28). In a recent study, FATP-1 knockout mice exhibited protection from fat-induced accumulation of intramyocellular fatty acyl-CoA and insulin resistance in skeletal muscle compared with wild type mice, despite lipid infusion or after a high-fat diet (29). Therefore, FATP-1-mediated lipid uptake is linked with lipid storage. Cellular exposure to insulin results in a rapid translocation of FATP-1 from an intracellular perinuclear compartment to the plasma membrane, which parallels LCACoA uptake (30, 31). This suggests that FATP-1 may be involved in insulin-mediated regulation of fatty acid uptake, particularly in obese non–insulin resistant conditions.

**Mitochondrial abnormalities**

One consistent finding with obesity is the reduced capacity for lipid oxidation through lowered activity of key mitochondrial enzymes (22, 32). Carnitine palmitoyl transferase is a particularly important enzyme responsible for fatty acid transport into the mitochondria. Reduced carnitine palmitoyl transferase activity has been consistently observed in obese volunteers (22, 33). Diminished activity of mitochondrial NAD (NADH) oxidoreductase, an enzyme that reflects the overall activity of the respiratory chain, has also been shown to occur in obese non-diabetic and T2DM patients relative to lean subjects (34). This is rather significant, because normal mitochondrial function is required for adequate cellular glucose and fatty acid metabolism and homeostasis. Petersen et al (35) found that diminished muscle insulin sensitivity associated with elevated intramyocellular triacylglycerols in elderly individuals corresponded with decreases in both mitochondrial oxidative capacity and mitochondrial ATP synthesis. The influence that skeletal muscle mitochondria plays in lipid turnover may in part explain why athletes have such high IMTG concentrations and yet are quite insulin sensitive, a finding vastly different from obese and diabetic patients. Endurance training in particular is known to increase both mitochondrial quantity and quality in skeletal muscle (see “Exercise modulation of skeletal muscle insulin sensitivity and lipid metabolism” section). Fatty acids in trained skeletal muscle are in a higher state of flux, and greater IMTG concentrations here may represent an adaptive response to training that is associated with greater insulin sensitivity. In contrast, obese persons and persons with T2DM exhibit reduced mitochondrial efficiency and lipid turnover, which may facilitate the build up of deleterious lipid metabolites and encourage lipid peroxidation, which in turn can affect both insulin signal transduction and mitochondrial function (34, 36). Overall, reduced lipid turnover is a necessary component to any apparent lipotoxic effects on insulin signaling that may arise from IMTG accumulation. Under conditions of reduced lipid oxidation, there is an increased load of fatty acids on the mitochondrial membrane facilitating the entrance of neutral fatty acids into the mitochondrial matrix (37), where they are prone to lipid peroxidation. Studies have shown that there is a higher degree of lipid peroxidation within skeletal muscle of obese insulin-resistant persons (36). The peroxide products are highly reactive cytotoxic metabolites that damage DNA and proteins and further hinder mitochondrial oxidative capacity. This constitutes a vicious cycle, and it is currently unclear whether mitochondrial defects lead to IMTG accumulation or whether IMTG accumulation leads to mitochondrial defects. Regardless, it seems apparent that each can affect the other.

**LIPID DISTRIBUTION IN SKELETAL MUSCLE**

One of the difficulties when working with skeletal muscle is the challenge of completely separating out the extraneous fatty and fibrous tissue that tends to accompany the muscle specimen and, therefore, minimize contamination that can affect sample analysis. In this section, we briefly discuss lipid distribution within the muscle fiber (intramyocellular) as opposed to between these fibers (intermuscular) and place particular emphasis on the heterogenous nature of skeletal muscle. Additionally, we define total fiber area as the volume of a muscle fiber, which is a cylindrical cell $\approx 10–100 \mu m$ in diameter and 1–400 mm in length (38).

Early evidence for in vivo intramyocellular lipid distribution and insulin resistance relied on histologic and biochemical analysis of skeletal muscle biopsies. These techniques have a fundamental flaw in that they do not discriminate adequately between intramyocellular and intermuscular lipid deposition. Additionally, repeated measurements are difficult because of the invasive nature of muscle biopsies especially when examining children. More recently, noninvasive techniques, such as proton magnetic resonance spectroscopy ($^1$H MRS), have enabled a detailed analysis by their ability to distinguish between protons of lipids outside the myofiber and those of intracellular lipids in humans (39–41).

**Lipid stores and muscle fiber type**

Previous studies have indicated that in obese skeletal muscle, intracellular lipid comprises $\approx 3–4\%$ of total fiber area, whereas in the muscle of lean persons, this value decreases to $\approx 1–2\%$ (17, 42). This represents a substantial difference in total body lipid distribution, given the percentage of whole-body mass that skeletal muscle makes up. For reference, skeletal muscle mass tends to make up $\approx 36.5\%$ of body weight (43), and, therefore, if one were to make a comparison between an obese man (100 kg) and a lean man (68 kg), this could amount, on average, to a 0.9 kg difference in myocellular lipid content between the two. Additionally, this difference may also be dependent on muscle fiber type (44–46). Skeletal muscle is a heterogenous tissue composed of 2 main distinct fiber categories, each with slightly different metabolic capabilities. Type I, or slow twitch, fibers are predominantly oxidative and contain more mitochondria than do type II, or fast twitch, glycolytic fibers. Therefore, type I fibers are more efficient “fat burning” fibers. Type I fibers also seem to be more responsive to insulin, exhibiting greater insulin binding capacity and increased insulin receptor kinase activity and phosphorylation compared with type II fibers (44, 47–49). Additionally, whole-body glucose uptake and muscle glucose transport are positively associated with type I fibers (44–46). This is a particularly significant fact when examining muscle fiber composition and obesity. Obese persons tend to exhibit fewer type I
fibers and an increased percentage of type II fibers than do lean subjects (45). Studies have reported a negative association between adiposity and the relative percentage of type I fibers (47, 50, 51). Given these observations, it is likely that there is a relation between muscle fiber composition and obesity, a notion supported by further studies (52).

INSULIN-MEDIATED SIGNALING TRANSDUCTION AND GLUCOSE UPTAKE

Recent reviews have discussed our current understanding of insulin signal transduction, particularly in resistant states such as T2DM (53). Therefore, it will be discussed only briefly to illustrate insulin-mediated glucose metabolism and to provide clarity when discussing signaling defects affiliated with lipid oversupply.

The insulin receptor (IR) is a heterotetrameric tyrosine kinase receptor composed of two α and two β chains and belongs to a family of growth factor receptors (54). Insulin binding triggers autophosphorylation of the receptor, which creates a recognition motif for the binding domain of insulin receptor substrates (IRSs) (55). There are ≥13 different IRSs (IRS 1–6, Gab-1, Shc 1–3, p62 dok, APS, and Cbl/CAP) (56–58), which show little sequence homology yet are functionally linked (59). These proteins, particularly IRS-1 and -2 and Shc, are tyrosine phosphorylated upon binding to the activated IR, which leads to further recruitment of src homology 2 domain-containing proteins. IRS-1, and to a lesser extent IRS-2, recruit the src homology 2 protein phosphoinositide 3-kinase (PI3K) (60), which then catalyzes the formation of phosphoinositide lipids such as P(3,4,5)P3, which activates 3-phosphoinositide-dependent protein kinase (PDK) 1. This then phosphorylates and activates other kinases, such as atypical protein kinase C (aPKC) and Akt (also known as protein kinase B) that mediate the translocation of the skeletal muscle glucose transporter GLUT4 to the cell membrane. The molecular details linking aPKC and Akt with GLUT4 translocation are currently unknown; therefore, the mechanisms underlying the control of insulin metabolism are not yet completely understood. What is known, however, is that Akt phosphorylates glycogen synthase kinase 3, the enzyme that inactivates glycogen synthase via phosphorylation. Phosphorylation of glycogen synthase kinase 3 by Akt inactivates the enzyme and therefore promotes glycogen synthesis.

Furthermore, insulin is known to mediate gene regulatory events though Shc and the activation of the ras–mitogen-activated protein kinase pathway. The protein Grb-2 is bound by both IRS-1 and Shc upon insulin receptor binding and subsequent receptor substrate phosphorylation. This binding facilitates ras activation and the extracellular-regulated kinase (ERK) mitogen-activated protein kinase cascade, which in turn affects expression of genes involved in the metabolic and growth-promoting effects of insulin (61). The whole pathway is illustrated in Figure 2.

ALTERATIONS IN SKELETAL MUSCLE INSULIN SIGNALING ASSOCIATED WITH EXCESS LIPID ACCUMULATION

Serine/threonine phosphorylation of insulin receptor 1

Pan et al (12) first reported in 1997 that the IMTG concentration is associated with insulin resistance in humans. Since then, other studies have shown similar associations (8, 62–64). Note that the bulk of these studies examined sedentary populations that were either overweight or obese, diabetic, or had a family history of T2DM. To understand the defects in skeletal muscle insulin signaling that are known to occur in these populations and mechanistically link IMTG deposition to these defects, a molecular understanding of insulin resistance is needed. Insulin resistance in general has been associated with reduced tyrosine phosphorylation of IRS-1, leading to diminished activity of PI3K (60, 65). As described previously, when IRS-1 becomes tyrosine phosphorylated, it recruits a number of SH2-containing signal transducers such as PI3K. Although the mechanisms leading to diminished phosphorylation have not as of yet been determined, it is important to realize that IRS-1 contains numerous potential serine/threonine phosphorylation sites as well. Serine/threonine phosphorylation of IRS-1 has been implicated in diminished insulin action (60, 66). Specifically, the structural mechanism appears to involve IRS protein dissociation from the IR by inducing conformational changes, thereby impairing access to tyrosine phosphorylation sites (66–68). This may further facilitate IRS release from the intracellular complexes that maintain the proteins in close proximity to the IR (69). The result is reduced IRS-1 activation of PI3K and, consequently, diminished GLUT4 translocation to the membrane surface.

To decipher whether elevated plasma triacylglycerol concentrations are specifically implicated in altered IRS-1 signaling, Belfort et al (21) examined a dose-response effect of elevated plasma FFA concentrations, comparable to concentrations observed in obese and T2DM subjects, on insulin-mediated glucose disposal in lean, healthy subjects. The authors observed a significant reduction in IRS-1 tyrosine phosphorylation and PI3K activity, associated, in part, with increased IRS-1 serine phosphorylation in muscle biopsy samples (21). Similar effects were also seen in additional human and animal studies (70–72). Furthermore, elevated FFA concentrations within the muscle itself have been linked with increased serine phosphorylation of IRS-1 (73),
indicating one specific mechanism of fatty acid-induced insulin resistance.

Serine phosphorylation of IRS-1 occurs at specific serine residues (68, 74) and seems to be the result of increased activation of particular isoforms of PKCs such as PKCθ (71), a novel DAG-dependent PKC (19). DAG is a fatty acid metabolite (see “Possible lipid mediators involved in insulin resistance” section), and because chronically elevated plasma FFA concentrations correspond to increased lipid deposition within the muscle, the concentration of DAG intracellularly is expected to increase. This increase in DAG concentrations is associated with the blunting of insulin signaling (72) and, therefore, may offer a very plausible mechanism to lipid-alteration of IRS-1 activity.

PKC isoforms and lipid-induced insulin resistance

The metabolic effects caused by insulin are predominantly mediated by effectors downstream of PI3K, with the most notable among these being PKCs. The PKC family is composed of >10 isoforms, grouped into atypical, classical, and novel PKCs (nPKCs). The role of these various PKC isoforms in insulin resistance has been studied extensively because of their lipid-induced insulin resistance mechanism to lipid-alteration of IRS-1 activity. Increase in DAG concentrations is associated with the blunting of insulin signaling (72) and, therefore, may offer a very plausible mechanism to lipid-alteration of IRS-1 activity.

Atypical PKCs

Studies conducted in diabetic patients, obese human subjects, or both have shown defective activation of aPKCs due to impaired activation of PI3K (82–84). Activation of aPKCs is required for insulin-mediated GLUT4 translocation to the plasma membrane, which enables glucose uptake in skeletal muscle (85). Therefore, by inhibiting appropriate phosphorylation and subsequent activation of these aPKCs by PI3K, insulin-mediated glucose uptake is impaired. Despite impaired aPKC activation, phosphorylation of Akt has shown to be relatively unaffected under diabetic conditions (82–84, 86–88), which presents a paradox. One plausible explanation may involve PI3K-independent factors that may also facilitate Akt activation either by direct protein-protein interaction, phosphorylation via another kinase, or through indirect means (slowing degradation or dephosphorylation, which would otherwise lead to inactivation). Regardless, defective activation of aPKC in insulin resistance may be the result of impaired IRS-1–dependent PI3K activation or through poor responsiveness of PDK to PI3K-mediated PI(3,4,5)P3 production. Interestingly, impaired activation of aPKC by PI(3,4,5)P3 has been shown to occur in diabetic rats (89), humans with T2DM (83), and muscle culture preparations of obese subjects (90).

Novel mechanisms facilitating GLUT4 translocation by aPKC activation independent of PI3K have been found in both adipocytes and skeletal muscle cells of rodents in vitro (91, 92). Because glucose serves not only as a source of energy, but also as a regulator of physiologic processes, its role as a metabolic regulator, particularly in the pathology of insulin resistance with respect to ERKs, has been of interest. Bandyopadhyay et al (93) reported previously that glucose activates ERKs in adipocytes by a mechanism that is independent of glucose uptake and metabolism yet dependent on Grb2. PI3K-independent aPKC activation seems to depend on ERK activation as well as phospholipase D (PLD), a membrane associated enzyme that can generate phosphatic acid (PA) from phosphatidylcholine (PC) (91). PA is a known direct activator of aPKCs (94, 95), and evidence has linked PLD-generated PA with GLUT4 translocation (92, 96, 97); however, this remains controversial. Millar et al (98) reported that inhibition of PA production by butanol did not affect insulin-mediated glucose uptake in 3T3-L1 adipocytes. In contrast, the concentration of butanol typically used for experiments such as this may not have been enough to effectively block PA production, and, therefore, PLD-dependent GLUT4 translocation would not have been effectively inhibited (99).

Regardless, insulin activation of PLD has been suggested to be mediated by ADP ribosylation factor (ARF) proteins, which are thought to regulate the synthesis of PLD products (100). ARF activation seems to be facilitated by specific guanine nucleotide exchange factors, in particular members of the cytoesin/ARF nucleotide-binding site opener (ARNO) family. ARNO-mediated recruitment of ARF proteins to the plasma membrane with insulin stimulation has been shown, suggesting a general model of PLD activation (100). On insulin binding, ARNO is translocated to the plasma membrane and interacts directly with the IR. The particular intricacies leading up to this interaction possibly involve direct interaction with specific protein binding domains on ARNO or through as of yet unidentified targets. More research is needed to clearly elucidate the role of ARNO and PLD activation of aPKC in response to insulin stimulation, especially with regard to high intracellular lipid concentrations.

In summary, defective activation of aPKCs appears to be implicated in insulin resistance, yet whether elevated intramyocellular triacylglycerol concentrations directly contribute to this impairment remains to be clarified. The insulin signaling pathway itself is a complex myriad of reactions and, therefore, factors that affect upstream effectors, such as the insulin receptor or IRS, will most likely translate to impaired activation of downstream targets. Indeed, defective aPKC activation may, in part, be the result of impaired IRS-1–dependent PI3K activation, which is itself affected by fatty acid metabolites.

Novel and classical PKCs

Several studies have shown that abnormal activation of nPKCs and cPKCs result in diminished insulin responsiveness, particularly in acute lipid accumulation (71, 101–106). Most studies have shown a positive correlation between intramyocellular lipid deposition and nPKC activity, particularly the serine kinases.
PKC\(\theta\) and PKCe. In rodent skeletal muscle, chronic activation of the nPKCs \(\delta, \epsilon, \text{ and } \theta\) was shown with high-fat feeding and was correlated with an increase in both intramyocellular lipid accumulation and DAG concentration (106). In humans, PKC\(\theta\) translocation was associated with insulin resistance in muscle after acute FFA infusion (71). PKCe was shown to mediate IR degradation and signal attenuation in vitro, whereas overexpression of IRS-1 in skeletal muscle may be linked with insulin resistance in the diabetic sand-rat Psammomys obesus upon high energy intake (101).

Because PKC\(\theta\) and PKCe are serine kinases, their heightened activation may lead to enhanced serine phosphorylation of IRS-1, which may interfere with IRS-1 tyrosine phosphorylation, thereby inhibiting PI3K activation (107). Yu et al (72) observed a 30% reduction in insulin activation of IRS-1 tyrosine phosphorylation and approximately a 50% reduction in IRS-1–associated PI3K activity after lipid infusion in rats coinciding with activation of PKC\(\theta\) activity. It was proposed that this was due to IRS-1 phosphorylation of serine\(^{307}\), a critical residue in IRS-1 inactivation as shown by Aguirre et al (108); they showed that mutation to alanine\(^{307}\) resulted in protection from tumor necrosis factor \(\alpha\) (TNF-\(\alpha\))–induced insulin resistance. An increase in membrane-associated PKC\(\theta\) accompanied by a decrease in the inactive cytosolic pool has been observed in lipid-induced insulin resistance (106, 109), possibly indicating increased mobilization of the kinase and subsequent phosphorylation of membrane-bound substrates. Alternatively, PKCe has been shown to be relatively resistant to proteolysis after long-term chronic activation under similar conditions (105, 106, 109, 110). Therefore, one may suspect that the role of both PKCs in lipid-mediated insulin desensitization are dependent on time: resistance seen with acute lipid infusion may be PKC\(\theta\)-mediated, whereas, over the long term such as that seen with chronic high-fat feeding, PKCe may be implicated. Because obesity is a chronic disease associated with elevated plasma FFA and potentially with significant lipid deposition in the muscle, both PKC isoforms may play a prominent role in the pathology of intramyocellular lipid deposition leading to insulin resistance. This is perhaps debatable, however, given that Kim et al (111) showed no change in skeletal muscle PKCe expression in a PKCnull mouse model that showed protective effects from lipid-induced insulin resistance. This suggests that it may be unlikely that other PKC isoforms significantly contribute to the protection of lipid-induced insulin resistance caused by the PKC\(\theta\) knockout.

Therefore, the role nPKCs play in lipid-mediated insulin resistance needs to be further examined. PKC\(\theta\) inactivation has lead to mixed results. Kim et al (111) showed that 3–4 mo-old PKC\(\theta\) knockout mice had normal glucose uptake and insulin-associated IRS-1 tyrosine phosphorylation and subsequent PI3K activation after a 5-h lipid-heparin infusion compared with abnormal levels of these variables observed in wild type mice. In contrast, a prior study by Serra et al (112) showed PKC\(\theta\) dominant negative mice had an age-associated reduction in insulin sensitivity and developed obesity at 6–7 mo of age. The discrepancy may be due to differences between PKC\(\theta\) deletion and dominant negative expression or it may be an issue of time. At 3–4 mo of age, obesity was not present, therefore providing the possibility that the reduced glucose tolerance and skeletal muscle insulin signaling seen in the dominant negative PKC\(\theta\) by age 6–7 mo may be secondary to obesity.

Unfortunately, little has been done to closely examine the effects of a PKCe knockout model specifically on lipid-mediated insulin resistance. However, other PKC knockout models have been examined. In particular, Standaert et al (110) examined cPKC \(\alpha\) and \(\beta\) knockout mice. Overall, glucose homeostasis in vivo was not impaired in PKC\(\beta\) knockout mice and although glucose transport did increase moderately in some tissues, PKC\(\beta\) was not considered essential to insulin-stimulated glucose transport (113). Similar findings were also reported for PKCe. PKCe was not required for insulin-stimulated glucose transport, yet activation of the kinase resulted in significant increases in this transport most likely due to insulin-induced activation of PI3K (114). A general picture of the PKC-mediated effects on insulin signaling is shown in Figure 3.

**POSSIBLE LIPID MEDIATORS INVOLVED IN INSULIN RESISTANCE**

Intracellular triacylglycerols are relatively inert molecules, and, as such, IMTG concentrations may merely represent a surrogate marker for the potential build up of other lipid species within the muscle. In particular, metabolically active cellular LCACoAs are seen as better predictors of insulin sensitivity than triacylglycerols (115, 116). They are the activated form of intracellular FFAs produced by the action of acyl-CoA synthase and are recognized as signaling molecules that participate in a variety of cellular processes and through these processes possibly influence skeletal muscle insulin action.

**Diacylglycerol and ceramide**

DAG and ceramide are intracellular fatty acid metabolites that have been suggested to play roles as primary mediators in lipid-induced insulin resistance (72, 117–119). Both are elevated in obese skeletal muscle with increased myocellular lipid content and have been shown to accumulate in insulin-resistant tissues (120). Therefore, considerable attention has been given to the effects of each molecule on insulin signaling.

DAG is an intermediate of both triacylglycerol and phospholipid metabolism that has been shown to accumulate in many human and rodent models of insulin resistance, including lipid-induced insulin resistance (105, 106, 109, 121–124). DAG can be generated by the breakdown of phospholipids via phospholipases or through de novo synthesis via the esterification of LCACoA to glycerol-3-phosphate (Figure 4). It acts as an important second messenger involved in intracellular signaling and, because of its role in cPKC- and nPKC-mediated activation (125, 126), is a prime candidate in lipid-induced insulin resistance. High DAG concentrations seem to correspond to greater IR and IRS-1 inhibition in animals (101), and chronic activation of nPKC \(\theta\) and \(\epsilon\) have been observed concomitantly with elevated DAG concentrations in high-fat fed rats. This activation was associated with insulin resistance (106). Furthermore, muscle cell studies have directly shown that DAG reduces insulin-stimulated glucose uptake by a PKC-dependent mechanism (118). Thus, it is possible that normalization of DAG concentrations ameliorate this aberrant PKC activity, potentially improving skeletal muscle insulin sensitivity through enhanced IRS activity.

Ceramide is a derivative of sphingomyelin, a phospholipid component of cell membranes, and is generated either by sphingomyelinase or via de novo synthesis with palmitoyl-CoA as the
precursor (Figure 4). Similar to DAG, ceramide can act as a second messenger either by altering the activity of kinases, phosphatases, or transcription factors and has been shown to play a role in cell proliferation, differentiation, and apoptosis (127). Insulin action has been shown to be inhibited by ceramide through inhibition of insulin signal transduction in vitro (119, 128–130). This may be due to the fact that Akt activation has been shown to be reduced in the presence of ceramide (129, 131), which in turn may lead to both reduced GLUT4 translocation to the plasma membrane and diminished glycogen synthase activity. In addition, overexpression of acid ceramidase, which catalyzes the lysosomal hydrolysis of ceramide to sphingosine and FFA, reversed the inhibitory effects that saturated FFAs have on insulin signaling by blocking their stimulation of ceramide accumulation (132).

**Peroxisome proliferator activated receptors**

Peroxisome proliferator activated receptors (PPARs) α, δ, and γ belong to a family of nuclear hormone receptors that regulate the expression of genes involved in glucose and lipid metabolism. They are bound and activated by fatty acids, their derivatives, or both. Elevated intramyocellular lipids are known to affect PPAR gene expression and, in turn, alter cellular metabolism [reviewed by Ferre (133)]. Furthermore, PPAR agonists have emerged as important pharmacologic treatments to improve hyperlipidemia and insulin action (134). Because PPARs regulate skeletal muscle fatty acid utilization, they merit further investigation.

PPAR-α is expressed in skeletal muscle and in other tissues such as liver, whereas PPAR-γ is mainly localized to adipose tissue and immune cells. PPAR-δ is ubiquitously expressed in all tissues. Insulin sensitivity seems to be significantly improved on PPAR-α activation in genetic (obese Zucker fa/fa rats), nutritional (high-fat diet), or lipoatrophic (A-ZIP/F-1) models of insulin resistance (135–137). PPAR-α activation increases lipid oxidation, thus reducing fatty acid content in tissue and minimizing lipotoxicity. The role of PPAR-γ in regulation of insulin resistance in skeletal muscle is not fully known. Pharmacologic ligands of PPAR-γ such as thiazolidinediones cause enhanced glucose disposal. Although PPAR-γ is localized mainly to white and brown adipose tissue and, to a lesser extent, immune cells, it

**FIGURE 3.** Protein kinase C (PKC)–mediated effects on insulin signaling. The left panel symbolizes normal insulin-mediated GLUT4 transport via activation of atypical PKC (αPKC). The right panel symbolizes insulin resistance due to aberrant activation of novel PKCs (nPKCs), which serine-phosphorylate the insulin receptor, thereby inhibiting the tyrosine autophosphorylation required for insulin receptor substrate (IRS) docking. Incidentally, IRSs are also serine phosphorylated by nPKCs (not shown). ARNO, cytohesin/ARF nucleotide-binding site opener; PLD, phospholipase D; PC, phosphatidylincholine; PA, phosphatidic acid; ARF, ADP ribosylation factor; PI3K, phosphoinositide 3 kinase; DAG, diacylglycerol; PDK, 3-phosphoinositide-dependent protein kinase.

**FIGURE 4.** De novo synthesis of diacylglycerol (DAG) and ceramide from fatty acids.
may play an indirect whole-body role in lipid-mediated insulin resistance. Expression of PPAR-γ in C2C12 skeletal muscle cells affects insulin sensitivity, which indicates the possibility of cross-talk between PPAR-γ and insulin in skeletal muscle cells (138). The exact connection remains in question, but it is possible that PPAR-γ activation encourages fatty acid channeling to adipose tissue, thereby reducing their availability to muscle by decreasing circulating FFAs. PPAR-δ is the predominant isoform in rodent skeletal muscle and, similar to PPAR-α, promotes fatty acid oxidation and utilization. Furthermore, PPAR-δ may be the main isoform mediating the response to increased fatty acid availability in muscle cells. Agonists of PPAR-δ seem to normalize blood lipids and reduce insulin resistance and adiposity in both rodents and primates. The PPAR-δ agonist GW501516 significantly increased fatty acid oxidation in C2C12 myotubes (139). It should be noted, however, that a recent study examining the effects of GW501516 in skeletal muscle tissue showed that, in contrast to cultured myotubes, no effect was seen with respect to glucose transport or enhanced insulin action (140). Therefore, although these PPAR-δ agonists show promise, more research is needed in vivo to determine their true effectiveness.

Inflammatory mediators

Subacute low-grade inflammation is associated with insulin resistance and T2DM, and various inflammatory mediators seem to be involved in lipid-mediated insulin desensitization. For example, abnormal activity of IκB kinase-β (IKK-β), a serine kinase of IRS-1, signifies a subacute inflammatory condition and has been clearly shown in insulin resistant states. Inhibition or normalization of IKK-β can prevent fat-induced insulin resistance [reviewed by Perseghin et al (141)]. Additionally, mice expressing constitutively active IKK-β in hepatocytes have a T2DM phenotype, including effects in the muscle that parallel those of high-fat fed wild-type mice (142). This insulin resistance associated with subacute inflammation from hepatic and muscle activation of IKK-β was reversed by inhibition of IKK-β (142). TNF-α has been shown to decrease insulin responsiveness in skeletal muscle by reducing IRS-1 and subsequent PI3K activity (143, 144) and by downregulating GLUT4 (145). Additionally, concentrations of skeletal muscle TNF-α in insulin-resistant obese patients have been shown to be 4-fold those of healthy volunteers (146). The inhibitory effects that TNF-α has on insulin signaling may also be attributed in part to the activation of sphinogomyelinase, which leads to the release of ceramide (147). Therefore, inhibition of IKK-β may hold potential for future pharmacologic treatments in obesity-induced insulin resistance.

One of the most prominent cytokines to be examined is interleukin 6 (IL-6). Its role in insulin resistance is controversial. Animal studies suggest that IL-6 can induce insulin resistance (148), and, in humans, circulating IL-6 may (149, 150) or may not (151, 152) be associated with diminished insulin sensitivity. Obese diabetic and nondiabetic patients show increased circulating IL-6 concentrations that correspond with reduced insulin sensitivity (153), yet it should be noted that, during resting conditions, 10–35% of the body’s IL-6 is produced by adipocytes (154). Within adipocytes, IL-6 production is linked with reduced insulin sensitivity, and its release can be triggered by TNF-α (155, 156). Its regulation in skeletal muscle is complex and not completely understood. A marked increase in circulating IL-6 concentrations has been shown to occur after exercise; this increase was mostly mediated by skeletal muscle (157–159). The contracting muscle fibers seem to produce and release IL-6, which induces several metabolic effects. IL-6 here induces lipolysis and fat oxidation and plays a role in glucose homeostasis during exercise (160, 161). This, therefore, suggests that IL-6 may play multiple roles in skeletal muscle.

THE INFLUENCE OF DIETARY FATTY ACID COMPOSITION ON SKELETAL MUSCLE LIPID DISTRIBUTION AND INSULIN SENSITIVITY

Although development of T2DM is linked to genetic predisposition, diet is a major contributor. The fatty acid composition of the diet and the relation it has with insulin resistance is currently a topic of intense investigation. Most observational studies suggest that certain fatty acid types promote insulin resistance, whereas other fatty acid types may protect against it. For example, high dietary intake of the monounsaturated fatty acid oleic acid, which is abundant in olive oil, has been associated with improved insulin sensitivity in the general population, whereas saturated fatty acids promote the opposite (162, 163). However, in observational studies such as these, it is difficult to distinguish between the effects of fat composition and the effects of energy density. Furthermore, no method of measuring dietary intake is completely reliable. Therefore, definitive evidence linking fat quality with insulin sensitivity and, additionally, IMTG accumulation can only truly be determined by intervention trials. Unfortunately, most trials (164–166) have been short term and have had a small number of subjects, and their results are inconclusive. Despite the lack of conclusive data from human studies, a substantial body of literature of studies that used animal models clearly suggests that certain fats promote skeletal muscle insulin resistance, though the effect specific fatty acids have on IMTG quantity is not clear. It is possible that high intakes of saturated fatty acids encourage IMTG accumulation compared with unsaturated fatty acid–rich diets, given that these latter fatty acid types are preferentially oxidized over the former (167). The overall significance of this, however, is speculative at the moment. Regardless, these animal studies suggest 2 possible ways in which fatty acid quality may affect insulin sensitivity. The first is with regard to sarcolemma fatty acid composition. In humans, as in other species, the body is particularly efficient at regulating the components of cell membranes such as the sarcolemma. However, the fatty acid composition of cellular membranes can be influenced by diet (168). This is especially of note, because the fatty acid types taken in by the human diet—saturated, monounsaturated, polyunsaturated, and trans-unsaturated fatty acids—differ by spatial configuration and thus chemical property, which in turn can affect cell membrane fluidity and rigidity. Overall, most animal and cell studies seem to indicate that saturated and trans-unsaturated fatty acids significantly increase insulin resistance, whereas polyunsaturated n–3 fatty acids improve it (169). The effects on insulin sensitivity of n–6 polyunsaturated fatty acids appear to range somewhere between the saturated and n–3 fatty acids (170). Because cellular membranes are complex networks in and of themselves, whereby the efficiency of molecular signal transduction is highly dependent on the orientation and positioning of various proteins within the membrane, the fatty acid composition of cellular membranes may play a pivotal role in an adequate insulin response. Cross-sectional studies conducted in humans suggest that the fatty acid composition of
phospholipids in the sarcolemma may modulate insulin sensitivity (171, 172), and, interestingly, obese patients or those with T2DM display a different fatty acid composition of serum lipids compared with lean subjects, with a higher proportion of the saturated fat palmitate and lower concentrations of linoleic acid (an n–6 fatty acid) (173). This may be due to differences in the quality of fat that each group tends to consume on a daily basis. Animal studies seem to directly show that saturated fat-laden membranes promote insulin resistance, whereas more unsaturated membranes protect against it, a finding also noted in humans (174).

Along with diet-induced changes in membrane fatty acid composition, the type of fat consumed seems to determine the fatty acid composition of the IMTG pool. In humans, insulin resistance directly correlates with increased saturated fatty acids in skeletal muscle triacylglycerols (175). Repeatedly, the quantity of IMTG seems not to be an entirely accurate maker for insulin sensitivity. Rather, IMTG accumulation in obese and T2DM skeletal muscle may represent more of a potential for the accumulation of specific lipid metabolites that in turn may negatively affect insulin sensitivity. The identity of these lipid metabolites may, to a certain extent, be influenced by the dietary fat composition of an individual’s diet. Animal studies have linked high saturated fatty acid intake with elevated concentrations of specific lipid messengers in muscle (176), and cell culture studies have been particularly insightful in directly linking a particular fatty acid type with a specific second messenger. For example, saturated FFAs, such as palmitate (16:0), stearate (18:0), or arachidate (20:0), effectively induce DAG and ceramide synthesis as well as inhibit Akt activation (117, 119, 177). Saturated FFAs with hydrocarbon chains shorter than those of palmitate do not produce these results (177). Because ceramide, for the most part, is derived from long-chain saturated fats, this may partially explain these findings. Palmitate is often used as the FFA of choice in these studies, because it is one of the most prevalent FFAs in plasma (in addition to the monounsaturated fat oleate). Saturated fatty acids, which accumulate in the form of DAG and activate PKC, have been shown to reduce glucose uptake via desensitization of insulin stimulation in cultured human skeletal muscle cells (118), and, as indicated previously, palmitoyl-CoA is a direct precursor to de novo ceramide synthesis. However, palmitate does not necessarily have to be converted to an intracellular lipid second messenger such as ceramide to affect intracellular signaling. Elevated palmitate concentrations can affect cellular signaling by inhibiting IR or IRS-1 phosphorylation (178) and Akt activation (117, 119, 177). This fatty acid has also been shown to induce cytokine expression (179) and result in downregulation of GLUT4 content (180, 181, 182). This can be explained on a molecular level on the basis of studies in which n–3 PUFAs prevented some of the aberrations seen with high intramyocellular lipid deposition. For example, one study showed that rats fed a high-fat diet enriched with n–3 fatty acids maintained the activity of IR, IRS-1, and PI3K activity, as well as total GLUT4 content in skeletal muscle (183). Additionally, n–3 PUFAs, specifically eicosapentaenoic acid and docosahexaenoic acid, which are found mainly in fatty fish, may reduce the rate at which insulin resistance progresses to T2DM (184, 185). Higher concentrations of n–3 PUFAs in the membrane of skeletal muscle are associated with lower fasting plasma glucose concentrations in rodents and humans [reviewed by Lombardo and Chicco (186)]. Furthermore, dietary intake of n–3 PUFAs in rats has been shown to induce an increase in the glucose-6-phosphate pool that is accompanied by an increase in glycogen synthesis, signifying enhanced glucose uptake (181). Generally, n–3 PUFAs seem to prevent the decrease of PI3K activity and minimize the GLUT4 depletion in skeletal muscle that would normally occur in lipid-induced insulin desensitization. Finally, it is interesting that n–3 PUFAs are preferentially oxidized over saturated fatty acids (167). These fatty acid types can modify fuel partitioning within the cell and upregulate genes involved in lipid oxidation such as PPARs and therefore possibly discourage IMTG accumulation (187).

With mounting evidence supporting the benefits of increased n–3 PUFA dietary intake, the American Diabetes Association has recommended the public consume 2–3 servings of fish rich in n–3 PUFAs per week (188). Unfortunately, despite the benefits that n–3 PUFA intake may have, there is still no definitive proof that these fats can actually reverse insulin resistance. Most intervention trials have not shown any benefit from consuming these fatty acid types in T2DM patients (189–191). Rather, their use should be a part of a healthier diet geared toward prevention of T2DM. Regardless, fish oil seems to have many beneficial effects in healthy subjects such as decreasing plasma triacylglycerols; therefore, increasing one’s dietary intake should prove helpful.

The beneficial effects seen with n–3 PUFAs have not been observed consistently with n–6 PUFAs; their effect on lipid-mediated insulin resistance in skeletal muscle has been mixed. For example, the n–6 PUFA arachidonic acid was seen as fairly effective in preventing alloxan-induced diabetes in male Wistar rats (192), and treatment of genetically diabetic GotoKakizaki rats with arachidonic acid significantly improved insulin sensitivity (193). Results seen with lipid-induced insulin resistance have been less impressive, however. Marotta et al (194) showed a significant increase in intramyocellular triacylglycerol concentrations in rats given a hypercaloric diet rich in n–6 PUFAs (sunflower oil). In contrast, little effect in these muscle lipid concentrations was seen when the n–6 PUFAs were substituted with either saturated or monounsaturated fat (194). Another study showed that a diet rich in n–6 PUFAs led to blunted signaling of IR and IRS-1 tyrosine phosphorylation and seemed to inhibit PI3K activity as well as reducing GLUT4 protein content (183). In comparison, this same study showed that a diet rich in n–3 PUFAs in addition to the n–6 PUFAs completely maintains insulin sensitivity, offsetting these effects. Lipid-induced stimuli that lead to c-JUN NH2-terminal kinase (JNK) activation, such as the n–6 fatty acid linoleate (195), seem to inhibit IRS-1 function though serine phosphorylation, thereby interrupting the IRS–insulin receptor interaction (196) or promoting IRS protein degradation (197). Gao et al (195) found that activation of PKCθ contributes to JNK activation and that JNK mediates PKCθ signals for serine phosphorylation and degradation of IRS-1. Conversely, skeletal muscle PKCθ translocation to the membrane induced by high-fat feeding in rats was reversed by...
across dietary manipulation, specifically feeding of a high-fat and low-fat meal (198).

On a final note, high dietary intake of trans-fatty acids seems to be associated with various deleterious effects, similar to intake of saturated fatty acids. Although small amounts of trans-fatty acids are present in nature, the artificial processing of unsaturated fats via the addition of hydrogen creates a chemically stable lipid that is currently used in a wide variety of processed foods. These hydrogenated lipids differ from their cis-unsaturated counterparts by spatial configuration, possessing the straight uninked structure similar to saturated fatty acids yet still display a degree of unsaturation. Little has been done to examine the effect that these fats have on lipid-mediated insulin resistance seen in skeletal muscle. One study that examined whether cis and trans-fatty acids of the same length acutely influence insulin release and glucose oxidation in isolated mouse pancreatic islet cells found that the trans-fatty acids elicited a greater insulin output than did their cis counterparts and additionally the cis isomers significantly inhibited glucose oxidation compared with the trans-fatty acids (199). Another study that compared both saturated and trans-fatty acids with monounsaturated fatty acids in healthy subjects showed no difference in insulin sensitivity and glucose oxidation (200). The literature overall seems to suggest that trans-fatty acids have no significant effect on insulin sensitivity in lean, healthy persons (200, 201), yet an elevated insulin response may occur in persons with T2DM (202). Large randomized controlled trials need to be done, and any possible link between these fatty acid types and specific lipid metabolites is unknown at this time.

EXERCISE MODULATION OF SKELETAL MUSCLE INSULIN SENSITIVITY AND LIPID METABOLISM

Endurance exercise improves skeletal muscle insulin sensitivity, and the mechanism of action is fairly well described. Notable points in skeletal muscle insulin signal modulation via this type of exercise include increases in GLUT4 protein concentrations and increased activities of both glycogen synthase and hexokinase, the enzyme that phosphorylates glucose (203, 204). As previously mentioned, endurance athletes are quite insulin-sensitive yet have high IMTG concentrations (17). Some studies have shown that placing sedentary adults on an endurance exercise program improves insulin sensitivity while increasing IMTG concentrations (16, 205). The effect of exercise is, of course, whole-body mediated, but in these studies, the improved insulin sensitivity in the presence of increased IMTG concentrations is most likely the result of more efficient lipid turnover in that the muscle is becoming more adept at lipid uptake, transport, utilization, and oxidation. Indeed, Menshikova et al (206) showed improvements in mitochondrial biogenesis and electron transport chain activity in older persons after 12 wk of endurance training. Bruce et al (14) obtained similar results in obese persons, although their IMTG concentrations remained relatively unchanged. Therefore, the capacity for lipid oxidation is increased, yet given the IMTG increase noted in some of these studies, greater FFA delivery and uptake must also be occurring (207, 208). The increase in lipid uptake most likely represents, again, an adaptation by the muscle to the increased metabolic demands that arise from strenuous physical exertion. This, coupled with increased FFA delivery to the exercising muscles, an expected physiologic response, would help to explain increased IMTG concentrations. The improvements in insulin sensitivity despite the increase in IMTG are likely related to reductions in deleterious lipid metabolites from a greater lipid flux. In the study by Bruce et al (14), obese subjects were exposed to endurance training, which yielded reductions in both intramyocellular DAG and ceramide content. Reductions in lipid metabolite concentrations may partly explain the improvements in GLUT4 translocation and activities of hexokinase and glycogen synthase. There is also some evidence suggesting that endurance training reduces susceptibility of skeletal muscle to lipid peroxidation (209). This may lead to further improvements in mitochondrial function. Lastly, the antiinflammatory effects of exercise are well known [reviewed by Petersen and Pedersen (210)], and studies have shown that exercise reduces TNF-α concentrations, which may in part explain the increases in GLUT4 expression.

In addition to endurance exercise, resistance training should also be regarded as an essential component in an individual’s daily lifestyle. From a physiologic point of view, it is well recognized that endurance exercise increases capillary density, improves blood flow to the muscles and skeletal muscle mitochondrial biogenesis, and enhances translational stability of key proteins involved in insulin signal transduction (203). However, endurance exercise does not substantially affect skeletal muscle hypertrophy and strength compared with resistance training. Because resistance training increases skeletal muscle mass (211), it can augment whole-body glucose disposal capacity (212–214). Furthermore, studies have shown that even a single resistance exercise training session can improve insulin sensitivity for up to 24 h after cessation of exercise (214–216) and that these benefits are possibly attributed in part to reductions in IMTG stores (217).

At first, this may seem contradictory to studies that have shown increases in IMTG from endurance exercise, which imply a discrepancy dependent on exercise type. However, it is important to distinguish between a single training session and multiple training sessions. Many studies examining a single endurance bout have also shown reductions in IMTG concentrations (218–220). It is widely agreed that to really achieve any substantial long-lasting benefit from physical exercise, the activity must be consistently repeated throughout one’s life. A single training session of either endurance or resistance exercise will undoubtedly lead to reduced IMTG concentrations, because these lipids have been shown to be a major fuel source in both exercise types, depending on the intensity of the exercise; though, admittedly, this is still rather controversial [reviewed by van Loon (221)]. The enhanced lipid turnover seen with endurance exercise (14, 222) is a consequential adaptation to the metabolic demands of the body. Unfortunately, studies on the metabolic demands of resistance training are rare. This is likely due to the methodologic difficulties associated with the non–steady state conditions of this type of exercise. Regardless, studies that use exercise, be it endurance or resistance training, have consistently shown improvements in skeletal muscle insulin sensitivity, and any so called “paradox” with regard to IMTG concentrations is explained when examining lipid turnover.

CONCLUSIONS

Insulin resistance is a highly complex condition, and the molecular details of its pathology have yet to be completely deciphered, particularly in relation to IMTG accumulation. Given the
overall mass of skeletal muscle coupled with its role in whole-body glucose homeostasis, understanding the etiology of insulin resistance in this particular tissue is important. IMTG deposition is deleterious when accompanied with reduced lipid turnover, as evident by the fact that endurance-trained persons have high IMTG concentrations yet are also quite insulin sensitive. Exercise improves insulin sensitivity by increasing the expression and activity of notable enzymes that are important to glucose uptake. Additionally, exercise improves lipid flux, and, therefore, high IMTG concentrations may represent an adaptive physiologic response to training. In contrast, obesity is associated with a reduced capacity for lipid oxidation, which by itself leads to IMTG deposition. However, it is unclear whether the reduced mitochondrial efficiency in obese skeletal muscle is a cause or consequence of IMTG deposition, because lipid peroxidation is known to induce mitochondrial damage. The quantity of IMTG in the obese state serves as a marker for the potential build up of specific lipid metabolites, the identity of which may be influenced by dietary fat composition. These metabolites have been shown to interfere with PI3K activation through activation of nPKCs that in turn lead to excessive serine phosphorylation of IRS. Additionally, the fatty acid composition of the sarcotlemma can also be influenced by diet, thus representing another means of affecting insulin sensitivity. However, human evidence that conclusively links dietary fat composition with both IMTG accumulation and insulin resistance is lacking, though both observational and animal studies are suggestive of an effect. n–3 Fatty acids seem to improve skeletal muscle insulin sensitivity, whereas saturated fats and possibly trans-unsaturated fats seem to do the opposite. Mixed results are often seen with n–6 fatty acids. Overall, it seems clear that a long-term exercise program, composed of both endurance and strength training, along with reductions in saturated fat intake, will prevent the occurrence of insulin resistance in the general population and improve insulin sensitivity in the obese population.

MPC completed the literature search and prepared the manuscript after feedback from SL-F and RAF. None of the authors have any financial or personal conflict of interest pertaining to this article.

REFERENCES


212. Miller WJ, Sherman WM, Ivy JL. Effect of strength training on glucose


Effect of dairy calcium or supplementary calcium intake on postprandial fat metabolism, appetite, and subsequent energy intake

Janne Kunchel Lorenzen, Sanne Nielsen, Jens Juul Holst, Inge Tetens, Jens Frederik Rehfeld, and Arne Astrup

ABSTRACT
Background: High calcium intake has been shown to increase fecal fat excretion.
Objective: Our aim was to examine whether a high calcium intake from dairy products or from supplements affects postprandial fat metabolism and appetite through fat malabsorption.
Design: Four different isocaloric meals were tested in 18 subjects according to a randomized crossover design. The test meals contained high (HC meal: 172 mg/MJ), medium (MC meal: 84 mg/MJ), or low (LC meal: 15 mg/MJ) amounts of calcium from dairy products or a high amount of calcium given as a calcium carbonate supplement (Suppl meal: 183 mg/MJ). Concentrations of plasma total triacylglycerol, chylomicron triacylglycerol, serum total cholesterol, HDL cholesterol, cholecystokinin, glucagon-like peptide 1, ghrelin, peptide YY, glucose, and insulin and appetite sensation were measured before and at regular intervals until 420 min postprandially.
Results: Dairy calcium significantly diminished the postprandial lipid response. The baseline adjusted area under the curve for chylomicron triacylglycerol was ≈17% lower after the MC meal (P = 0.02) and ≈19% lower after the HC meal (P = 0.007) than after the LC meal and ≈15% lower after the MC meal (P = 0.0495) and ≈17% lower after the HC meal (P = 0.02) than after the Suppl meal. No consistent effects of calcium on appetite sensation, or on energy intake at the subsequent meal, or on the postprandial responses of cholecystokinin, glucagon-like peptide 1, ghrelin, peptide YY, insulin, or glucose were observed.
Conclusions: Increased calcium intakes from dairy products attenuate postprandial lipemia, most probably because of reduced fat absorption, whereas supplementary calcium carbonate does not exert such an effect. This may be due to differences in the chemical form of calcium or to cofactors in dairy products. Calcium did not affect appetite sensation, glucose metabolism, or gut hormone secretion.  


INTRODUCTION
The possibility that a high calcium intake may affect energy balance has attracted considerable attention. Several observational studies have found inverse associations between calcium intake or intake of dairy products and body weight, composition, or both (1–6). On the basis of a reanalysis of data from 4 observational studies, Davies et al (1) concluded that differences in calcium intake could explain ≈3% of the variation in body weight. However, other observational studies have failed to find this association (7–9). Recently, relatively few intervention studies have been reported, and those that have been reported are ambiguous with respect to the effect of calcium intakes on body weight and body composition (1, 10–16). In addition, in both animal models and in human studies it has been found that calcium from dairy products has a more profound effect on body weight and composition than does supplementary calcium (6, 14). Many different mechanisms have been suggested to be responsible for the positive effect of a high calcium intake on energy balance. Zemel et al (6) suggested that calcium affects adipocyte metabolism and fat oxidation. Animal studies have found that dairy calcium increases fecal fat excretion (17). In a short-term intervention study, we recently showed that increases in daily calcium intake increase fecal fat and energy excretion, whereas we failed to find any effect on energy expenditure (18). An increase in calcium intake of ≈1300 mg/d, mainly from low-fat dairy products, caused a daily increase in fecal fat excretion of 8.2 g in subjects consuming a diet with 30% of energy derived from fat. Others have shown a similar effect, though quantitatively smaller (19–21). The difference in effects might have been due to the source of calcium or to the protein source (18). The subjects in our study

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2 Supported by the Danish Dairy Research Foundation, FOOD Graduate School, Arla Foods Ingredients amba, Pharma Vinci A/S, The Dutch Dairy Association, Programme Commission on Food and Health (Danish Research Agency), and the Danish Medical Research Council. The cacao butter was a gift from Aarhus United Denmark.
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were given calcium from dairy products, whereas the subjects in the other studies were given calcium supplements or fortified products. The increased fat excretion was presumably due to formation of insoluble calcium fatty acid soaps or to the binding of bile acids, which impairs the formation of micelles (22–25). When fat is absorbed, it enters blood circulation in the form of intestinally derived triacylglycerol-rich lipoproteins, i.e., chylomicrons. Thus, if calcium partly inhibits fat absorption, a decrease in the postprandial increase in chylomicron triacylglycerol would be expected. As far as we know, the effect of calcium on postprandial fat absorption has not been examined previously. Dairy calcium may produce weight loss by a mechanism other than fat malabsorption. Although we, and others, have failed to find any effect of calcium on 24-h energy expenditure during energy balance (18, 26), the effects of calcium on appetite regulation have been less well studied. It is also possible that the increased fecal energy loss produced by dairy calcium might be recognized by the body and result in a compensatory increase in appetite and calorie intake. The aim of the present study was to examine whether a high calcium intake from dairy products or from supplements affects postprandial fat metabolism and appetite.

SUBJECTS AND METHODS

Subjects

Eighteen male subjects were recruited through advertising at universities in Copenhagen and on the internet. The subjects were healthy, between 18 and 50 y old, and moderately overweight [body mass index (in kg/m²): 24–31]. The exclusion criteria were as follows: lactose intolerance, milk allergy, diabetes, hypertension, hyperlipidemia, smoking, elite athletes, regular use of medication, use of dietary supplements, blood donation within the 6 mo preceding the study, and a hemoglobin concentration <130 g/L. The subjects were instructed to not donate blood during the study period. All subjects gave written consent after having received verbal and written information about the study. The study was carried out at the Department of Human Nutrition, Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark, and was approved by the Municipal Ethical Committee of Copenhagen and Frederiksberg in accordance with the Helsinki-II declaration (KF 01-144/02). The subjects received 3000 Danish crowns (~US$500) on completion of all tests.

Experimental design

Four different isocaloric meals were tested in 18 subjects according to a randomized crossover design. The subjects were randomly assigned to the sequence of the test meals. There was a washout period of ≥3 wk between the test meals. Each test meal lasted 8.5 h. The subjects were instructed to not eat or drink dairy products or food items rich in fat, such as potato crisps or chocolate, for 2 d immediately before each test meal or to drink alcohol or perform hard physical activities on the day before each test meal. The subjects consumed a standardized meal supplied by the study, which consisted of a pasta dish, orange juice, and biscuits (4 MJ; 17.6% of energy from protein, 22.1% of energy from fat, and 60.3% of energy from carbohydrate) on the evening before the test. The subjects fasted after consuming this meal at no later than 2000. They were supplied with 0.5 L water and were instructed to consume half of it before they went to bed and the remaining half in the morning before leaving the house. On the morning of the tests, the subjects arrived at the Department of Human Nutrition by car, bus, train, or slowly walking (ie, the least strenuous means of transportation). On arrival they were weighed while wearing only underwear and after emptying their bladder. Weight was measured in kilograms with one decimal by a Lindetronic 8000 scale (Copenhagen, Denmark). A Venflon catheter (BD Infusion Therapy AB, Helsingborg, Sweden) was then inserted in an antecubital arm vein. After 20 min of rest, a fasting blood sample was taken and the subject’s appetite sensation was assessed with a visual analogue scale (VAS). The test meal was then served. The subjects were instructed to consume the meal within 15 min, after which they completed a questionnaire on the palatability of the meal. Blood samples were collected 15, 30, 60, 90, 120, 180, 300, and 420 min after the start of the meal, and appetite sensation was assessed every 30 min throughout the 7 h after the meal. During the day the subjects were allowed to read, walk around at the Department of Human Nutrition, listen to the radio, or watch television or videos. One to 3 subjects were tested on each test day. The subjects were allowed to talk with each other as long as the conversation did not involve food, appetite, or related issues. The subjects were not allowed to eat or drink during the day except for 2 glasses of water (250 mL each), which were served 180 and 300 min after the meal (after the blood samples were drawn). At the end of the day, after the last blood sample was taken, an ad libitum meal consisting of a pasta salad (15% of energy from protein, 30% of energy from fat, and 55% of energy from carbohydrate) and water was served, food intake was registered, and energy intake was calculated. The subjects were instructed to eat until “comfortable satisfaction.” Afterward, they completed a questionnaire on the palatability of the meal, and appetite sensation was assessed. The subjects’ water intake was registered on the first test day, and this intake was repeated on the following test days.

Test meals

Four isocaloric test meals were provided to the subjects in a randomized order on 4 different days (Table 1). The test meals contained dairy proteins as the main protein source and a high amount of calcium from dairy products (HC meal), a medium amount of calcium from dairy products (MC meal), a low amount of calcium from dairy products (LC meal), or a low amount of calcium from dairy products and a calcium supplement served as a drink containing calcium carbonate (Suppl meal).

The test meals consisted of a bread roll, dairy products, chocolate, and water (Table 1). All of the test meals consisted of the same servings of bread rolls, yogurt, and liquid. The content of macronutrients and micronutrients in each meal was estimated by using Dankost 3000 dietary assessment software (Danish Catering Center, Herlev, Denmark) (27) and information from Arla Foods Ingredients amba (Viby, Denmark). The calcium and energy contents were analyzed. The test meals had the same macronutrient composition: 15% of energy from protein, 39% of energy from fat, and 46% of energy from carbohydrate. In addition, all test meals contained the same amounts of lactose, the content of which was adjusted by adding lactose powder to the bread roll or the drink, and the same amounts of whey and casein, which were achieved with the addition of whey powder (Liprodan DI-9213; Arla Foods Ingredients amba) and casein powder (Miprodan 30; Arla Foods Ingredients amba).
TABLE 1  
The content and nutrient composition of the 4 test meals  

<table>
<thead>
<tr>
<th>Test meals</th>
<th>LC</th>
<th>MC</th>
<th>HC</th>
<th>Suppl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bread roll</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk (g)</td>
<td>0</td>
<td>0</td>
<td>75</td>
<td>0</td>
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<td>Water (g)</td>
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<td>75</td>
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<td>100</td>
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<tr>
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<td>122.5</td>
<td>122.5</td>
<td>122.5</td>
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<tr>
<td>Cacao butter (g)</td>
<td>49.25</td>
<td>48</td>
<td>47.25</td>
<td>49.25</td>
</tr>
<tr>
<td>Casein powder (g)</td>
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<td>5</td>
<td>0</td>
<td>27.75</td>
</tr>
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<tr>
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<td>31.5</td>
</tr>
<tr>
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<td>5.5</td>
<td>0</td>
</tr>
<tr>
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<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Yogurt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yogurt (g)</td>
<td>21</td>
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<td>54</td>
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<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
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<td>2.3</td>
<td>2.3</td>
<td>7.8</td>
</tr>
<tr>
<td>Drink</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk (g)</td>
<td>0</td>
<td>266</td>
<td>554</td>
<td>0</td>
</tr>
<tr>
<td>Water (g)</td>
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<td>255.5</td>
<td>0</td>
<td>463</td>
</tr>
<tr>
<td>Casein powder (g)</td>
<td>13.5</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Whey powder (g)</td>
<td>3.5</td>
<td>1.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lactose (g)</td>
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<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sugar (g)</td>
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<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Calcium supplement (g)</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Nutrient composition</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Energy (kJ)</td>
<td>4464</td>
<td>4146</td>
<td>4619</td>
<td>4649</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>14.9</td>
<td>14.9</td>
<td>14.9</td>
<td>14.9</td>
</tr>
<tr>
<td>Fat (% of energy)</td>
<td>39.3</td>
<td>39.1</td>
<td>39.3</td>
<td>39.3</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>45.9</td>
<td>45.9</td>
<td>45.8</td>
<td>45.9</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>68</td>
<td>350</td>
<td>793</td>
<td>850</td>
</tr>
<tr>
<td>Phosphate (mg)</td>
<td>521</td>
<td>1253</td>
<td>1913</td>
<td>521</td>
</tr>
<tr>
<td>Casein (g)</td>
<td>27.2</td>
<td>27.8</td>
<td>27.4</td>
<td>27.2</td>
</tr>
<tr>
<td>Whey (g)</td>
<td>4.0</td>
<td>4.2</td>
<td>4.3</td>
<td>4.0</td>
</tr>
<tr>
<td>Lactose (g)</td>
<td>34.2</td>
<td>34.9</td>
<td>34.8</td>
<td>34.2</td>
</tr>
</tbody>
</table>

1. LC, MC, and HC, low, medium, and high amounts of calcium from dairy products; Suppl, high amounts of calcium as supplementary calcium carbonate.
2. Lacprodan DI-9213; Arla Foods Ingredients amba.
3. Miprodan 30; Arla Foods Ingredients amba.
4. Lactose; Miprodan 30; Arla Foods Ingredients amba, Viby, Denmark.
5. Calcium carbonate; Pharma-Vinci A/S, Frederiksværk, Denmark.
6. Water; Miprodan 30; Arla Foods Ingredients amba, Viby, Denmark.

The test meals were prepared at the Department of Human Nutrition from normal Danish food items. Portions were matched to each subject’s individual energy requirement and adjusted to the nearest 1 MJ. Each test meal contained 50% of the subject’s daily energy requirement. The subjects’ energy requirements were determined by using the following formula, which is based on a meta-analysis of our 24-h calorimetry studies (B Buemann, unpublished observations, 2003):

24-h EE(kJ/d) =

\[
1780.6 + 101.2 \times FFM + 32.2 \times FM + 212.4 \times \text{SPA} + 4.03 \times \text{DE} - 4.17 \times \text{age}\] 

Spontaneous physical activity was 5.8% for men and duration of exercise (DE) was set to 30 min (28). Body composition was measured at baseline with the bioelectrical impedance method, and fat-free mass and fat mass were calculated.

The subjects’ habitual diet was assessed with the use of a 3-d weighed food record (2 weekdays and 1 Saturday). The subjects were given both verbal and written instructions on how to complete the food record, and digital kitchen scales were supplied. Dankost 3000 dietary assessment software (Danish Catering Center) (27) was used to calculate the energy and nutrient composition of the diets.

Visual analogue scale

Visual analogue scales, 100 mm in length with words expressing the most positive and the most negative rating anchored at each end, were used to measure subjective appetite sensation (hunger, satiation, fullness, prospective food consumption, thirst, well-being, and desire to eat meat or fish or something sweet, salty, or fatty) and palatability of the test and ad libitum meals (Table 2). The questions were provided in small booklets, one question at a time. The subjects were instructed to not compare their ratings with each other and could not refer to their previous ratings when filling in the visual analogue scale.

Analyses

Venous blood samples were drawn at baseline and 15, 30, 60, 90, 120, 180, 300, and 420 min after the test meal started. Blood samples for plasma analyses were collected in tubes containing EDTA and placed on ice immediately. Blood samples for serum analyses were collected in serum tubes and kept at room temperature for 30 min to coagulate. All samples were centrifuged at 2800 × g for 15 min at 4 °C and stored at −20 °C until analyzed (exceptions are described below).

Triacylglycerol

Triacylglycerol was measured in blood samples drawn at baseline and 60, 120, 300, and 420 min after the start of the test meal. The chylomicrons were isolated within 24 h by ultracentrifugation as described elsewhere (29). Plasma total triacylglycerol, chylomicron triacylglycerol, and triacylglycerol concentrations in the bottom fraction were measured with an enzymatic endpoint method (Test-Combination Triacylglycerol GPO-PAP kit; Roche, Basel, Switzerland) by using a Cobras Mira Plus (Roche). The intraassay and interassay CVs were 0.5% and 1.4%, respectively. The percentage of recovery was calculated, and the chylomicron triacylglycerol concentration was adjusted to the concentration corresponding to 100% recovery.

Plasma concentrations of glucose, cholecystokinin, ghrelin, glucagon-like peptide 1, and peptide YY

Plasma concentrations of glucose, cholecystokinin (CCK), ghrelin, glucagon-like peptide 1 (GLP-1), and peptide YY (PYY) were measured in blood samples drawn at baseline and 15, 30, 60, 90 (only glucose), 120, 180, 300 (except glucose), and 420 min after the start of the test meal. Glucose was measured with the use of an enzymatic endpoint method (Hexokinase) (Gluco-quant Glucose/HK, Roche Diagnostics, Basel, Switzerland) with a Cobras Mira Plus (Roche). The intraassay and interassay CVs were 0.5% and 1.4%, respectively. The percentage of recovery was calculated, and the chylomicron triacylglycerol concentration was adjusted to the concentration corresponding to 100% recovery.
TABLE 2
Questions on appetite, desire to eat specific foods, and palatability of the test meals

<table>
<thead>
<tr>
<th>Answer (0 mm)</th>
<th>Question</th>
<th>Answer (100 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I am not hungry at all</td>
<td>How hungry do you feel?</td>
<td>I have never been more hungry</td>
</tr>
<tr>
<td>I am completely empty</td>
<td>How satisfied do you feel?</td>
<td>I cannot eat another bite</td>
</tr>
<tr>
<td>Not at all full</td>
<td>How full do you feel?</td>
<td>Totally full</td>
</tr>
<tr>
<td>Nothing at all</td>
<td>How much do you think you can eat?</td>
<td>A lot</td>
</tr>
<tr>
<td>Yes, very much</td>
<td>Would you like to eat something sweet?</td>
<td>No, not at all</td>
</tr>
<tr>
<td>Yes, very much</td>
<td>Would you like to eat something salty?</td>
<td>No, not at all</td>
</tr>
<tr>
<td>Yes, very much</td>
<td>Would you like to eat something fatty?</td>
<td>No, not at all</td>
</tr>
<tr>
<td>Yes, very much</td>
<td>Would you like to eat meat or fish?</td>
<td>No, not at all</td>
</tr>
<tr>
<td>Not at all</td>
<td>How thirsty do you feel?</td>
<td>Very much</td>
</tr>
<tr>
<td>Not comfortable at all</td>
<td>How comfortable do you feel?</td>
<td>Very comfortable</td>
</tr>
<tr>
<td>Good</td>
<td>Visual appeal</td>
<td>Bad</td>
</tr>
<tr>
<td>Good</td>
<td>Smell</td>
<td>Bad</td>
</tr>
<tr>
<td>Good</td>
<td>Taste</td>
<td>Bad</td>
</tr>
<tr>
<td>Much</td>
<td>Aftertaste</td>
<td>None</td>
</tr>
<tr>
<td>Good</td>
<td>Overall palatability</td>
<td>Bad</td>
</tr>
</tbody>
</table>

Calcium and energy contents of the test meals

A portion of each meal was freeze-dried and homogenized. Gross energy content was obtained by using a bomb calorimeter (Ika-calorimeter system C4000; Ika, Heitersheim, Germany). The analyses were performed as duplicates. Before the calcium content was measured, the samples were lyophilized and microwave digested (MES-1000; CEM Corporation, Matthews, NC) with 65% HNO₃ (suprapur; Merck, Darmstadt, Germany) and 30% H₂O₂ (suprapur; Merck). Calcium was measured by atomic absorption spectroscopy (SpectraAA-200 VARIAN; Varian Techtron Pty, Limited, Victoria, Australia) after dilution with a lanthanium oxide solution (Merck). Standards were prepared from a 1000-mg Ca/L standard (Tritisol; Merck) by dilution with lanthanium oxide solution. A reference diet (Standard Reference Material 1548a; Typical Diet; National Institute of Standards and Technology, Gaithersburg, MD) was analyzed in the same run. The percentage of recovery of calcium in the reference diet was 99.7% ± 0.7% (n = 5).

GLP-1 concentrations in plasma were measured by radioimmunoassay after extraction of plasma with 70% ethanol (by vol, final concentration). Carboxy-terminal GLP-1 immunoreactivity was determined by using antiserum code no. 89390, which has an absolute requirement for the intact amidated carboxy-terminus of GLP-1 7–36 amide and cross-reacts <0.01% with carboxy-terminally truncated fragments, and 89% with GLP-1 9–36 amide, the primary metabolite of dipeptidyl-peptidase IV-mediated degradation (33). The sum of the 2 components (total GLP-1 concentration) reflects the rate of secretion of the L cell. Sensitivity was <5 pmol/L, and the intraassay CV was <10%.

Serum concentrations of insulin, total cholesterol, and HDL cholesterol

Serum concentrations of insulin, total cholesterol, and HDL cholesterol were measured in blood samples drawn at baseline and 30, 60, 90, 120, 180, 300 (except insulin), and 420 min after the start of the test meal. Insulin was measured by solid-phase, 2-site chemiluminescent immunometric assay (Immulite/immulite 1000 insulin; Diagnostic Products Corporation, Los Angeles, CA) with the use of an Immulite 1000 analyzer (Diagnostic Products Corporation). The intraassay and interassay CVs were 2.5% and 4.9%, respectively. Total cholesterol was measured with an enzymatic endpoint method (CHOD-PAP) (cholesterol kit; Roche) by using a Cobras Mira plus (Roche, Basel, Switzerland). The intraassay CV was 0.9%, and the interassay CV was 1.5%. HDL cholesterol was measured with an enzymatic colorimetric test (HDL cholesterol plus second generation; Roche) by using a Cobras Mira Plus (Roche). The intraassay and interassay CVs were 1.9% and 3.5%, respectively.
89%. The calcium content in the samples was adjusted to concentrations corresponding to 100% recovery.

Calculations and statistical methods

Data were analyzed with the use of SAS software (version 8; SAS Institute, Cary, NC). Data are reported as means ± 95% confidence limits (CLs) unless otherwise indicated. A P value <0.05 is considered significant.

Repeated-measures analysis of variance (ANOVA) was used to assess the effect of time, meal, and interaction of meal and time. To adjust for differences in baseline values, these factors were included as covariate in all analyses. The analysis was performed in PROC MIXED. Based on the structure of the covariance matrix, the Gaussian model of spatial correlation was chosen for covariance structure. The study had a crossover design, so “subject” was included as a random effect. We tested for a significant effect of sequence of the test meals, and sequence of the test meals was included as a covariate when this was the case.

Area under the curve (AUC) was calculated as the total increase above zero. Analysis of covariance (ANCOVA) was used to examine the effect of meal on AUC. ANCOVA was performed in PROC MIXED. “Subject” was included as a random effect and baseline values as a covariate in all analyses. The sequence of the test meals was included as a covariate when it was found to have a significant effect. ANOVA was used to examine the effect of meal on the palatability of the meals and ad libitum intake. ANOVA was performed in PROC MIXED, with subject included as a random effect. Model control for all analyses was performed in PROC MIXED, and data were transformed before analysis if necessary. Least-squares means were used to estimate the adjusted means. Pairwise comparisons of the least-squares means were performed by paired t test.

The recording of appetite sensation measured by VAS after intake of the ad libitum meal was not included in the calculation of AUC or in any of the analyses.

RESULTS

All 18 subjects completed the 4 test meals. The characteristics of the subjects and their habitual intake are presented in Table 3. Blood variables from one subject were excluded from the data analyses because of abnormal triacylglycerol values. The values in parentheses are for the 17 subjects included in these analyses.

Area under the curve (AUC) was calculated as the total increase above zero. Analysis of covariance (ANCOVA) was used to examine the effect of meal on AUC. ANCOVA was performed in PROC MIXED. “Subject” was included as a random effect and baseline values as a covariate in all analyses. The sequence of the test meals was included as a covariate when it was found to have a significant effect. ANOVA was used to examine the effect of meal on the palatability of the meals and ad libitum intake. ANOVA was performed in PROC MIXED, with subject included as a random effect. Model control for all analyses was performed in PROC MIXED, and data were transformed before analysis if necessary. Least-squares means were used to estimate the adjusted means. Pairwise comparisons of the least-squares means were performed by paired t test.

The recording of appetite sensation measured by VAS after intake of the ad libitum meal was not included in the calculation of AUC or in any of the analyses.

Plasma triacylglycerol response

The postprandial responses in plasma total and chylomicron triacylglycerol are shown in Figure 1. Plasma total and chylomicron triacylglycerol concentrations increased after intake of each of the 4 test meals. The concentrations peaked 180 min after intake of the MC and the HC meals and 300 min after intake of the LC meal and the Suppl meal. At the end of the study, the concentrations had decreased to values similar to the baseline concentrations (P < 0.007) when adjusted for baseline concentration. However, pairwise comparisons of the adjusted means showed no persistent effects. There was a significant effect of time on chylomicron triacylglycerol concentrations (P < 0.0001) when adjusted for baseline concentration.

There was no significant meal-by-time interaction and no significant effect of meal.

The AUC for plasma total and chylomicron triacylglycerol are shown in Figure 1. ANCOVA showed that meal had a significant effect on AUC for chylomicron triacylglycerol (P = 0.01) when adjusted for baseline concentration. The adjusted AUC for chylomicron triacylglycerol was lower after intake of the MC (≈17%; P = 0.02) and HC (≈19%; P = 0.007) meals than after the LC meals. Similarly, the AUC was lower after intake of the MC (≈15%; P = 0.0495) and the HC (≈17%; P = 0.02) meals than after the Suppl meal. There were no significant differences between the other meals. Meal had no significant effect on AUC for plasma total triacylglycerol adjusted for baseline concentration.

Serum cholesterol

Repeated-measures ANOVA of serum total cholesterol and of HDL cholesterol, both adjusted for baseline concentrations, showed no significant meal-by-time interaction and no significant effect of meal (data not shown). However, a significant effect of time on both serum total cholesterol (P = 0.01) and HDL cholesterol (P < 0.0001) was observed.

ANOVA showed that meal had no significant effect on the AUC for total cholesterol when adjusted for the baseline concentration of total cholesterol or on the AUC for HDL cholesterol when adjusted for the baseline concentration of HDL cholesterol and the sequence of the test meals (data not shown).

Plasma concentrations of glucose, cholecystokinin, ghrelin, glucagon-like peptide 1, and peptide YY

The postprandial responses of glucose, CCK, ghrelin, GLP-1, PYY, and insulin are shown in Figure 2. Repeated-measures ANOVA showed a significant meal-by-time interaction on GLP-1 (P = 0.007) when adjusted for baseline concentration. Comparisons of adjusted least-squares means showed that the GLP-1 concentration was higher 60 min after intake of the Suppl meal on the palatability of the meals and ad libitum intake. ANOVA was performed in PROC MIXED, with subject included as a random effect. Model control for all analyses was performed in PROC MIXED, and data were transformed before analysis if necessary. Least-squares means were used to estimate the adjusted means. Pairwise comparisons of the least-squares means were performed by paired t test.

The recording of appetite sensation measured by VAS after intake of the ad libitum meal was not included in the calculation of AUC or in any of the analyses.

There was no significant meal-by-time interaction and no significant effect of meal.

The AUC for plasma total and chylomicron triacylglycerol are shown in Figure 1. ANCOVA showed that meal had a significant effect on AUC for chylomicron triacylglycerol (P = 0.01) when adjusted for baseline concentration. The adjusted AUC for chylomicron triacylglycerol was lower after intake of the MC (≈17%; P = 0.02) and HC (≈19%; P = 0.007) meals than after the LC meals. Similarly, the AUC was lower after intake of the MC (≈15%; P = 0.0495) and the HC (≈17%; P = 0.02) meals than after the Suppl meal. There were no significant differences between the other meals. Meal had no significant effect on AUC for plasma total triacylglycerol adjusted for baseline concentration.

Serum cholesterol

Repeated-measures ANOVA of serum total cholesterol and of HDL cholesterol, both adjusted for baseline concentrations, showed no significant meal-by-time interaction and no significant effect of meal (data not shown). However, a significant effect of time on both serum total cholesterol (P = 0.01) and HDL cholesterol (P < 0.0001) was observed.

ANOVA showed that meal had no significant effect on the AUC for total cholesterol when adjusted for the baseline concentration of total cholesterol or on the AUC for HDL cholesterol when adjusted for the baseline concentration of HDL cholesterol and the sequence of the test meals (data not shown).

Plasma concentrations of glucose, cholecystokinin, ghrelin, glucagon-like peptide 1, and peptide YY

The postprandial responses of glucose, CCK, ghrelin, GLP-1, PYY, and insulin are shown in Figure 2. Repeated-measures ANOVA showed a significant meal-by-time interaction on GLP-1 (P = 0.007) when adjusted for baseline concentration. Comparisons of adjusted least-squares means showed that the GLP-1 concentration was higher 60 min after intake of the Suppl meal on the palatability of the meals and ad libitum intake. ANOVA was performed in PROC MIXED, with subject included as a random effect. Model control for all analyses was performed in PROC MIXED, and data were transformed before analysis if necessary. Least-squares means were used to estimate the adjusted means. Pairwise comparisons of the least-squares means were performed by paired t test.

The recording of appetite sensation measured by VAS after intake of the ad libitum meal was not included in the calculation of AUC or in any of the analyses.

There was no significant meal-by-time interaction and no significant effect of meal.

The AUC for plasma total and chylomicron triacylglycerol are shown in Figure 1. ANCOVA showed that meal had a significant effect on AUC for chylomicron triacylglycerol (P = 0.01) when adjusted for baseline concentration. The adjusted AUC for chylomicron triacylglycerol was lower after intake of the MC (≈17%; P = 0.02) and HC (≈19%; P = 0.007) meals than after the LC meals. Similarly, the AUC was lower after intake of the MC (≈15%; P = 0.0495) and the HC (≈17%; P = 0.02) meals than after the Suppl meal. There were no significant differences between the other meals. Meal had no significant effect on AUC for plasma total triacylglycerol adjusted for baseline concentration.

Serum cholesterol

Repeated-measures ANOVA of serum total cholesterol and of HDL cholesterol, both adjusted for baseline concentrations, showed no significant meal-by-time interaction and no significant effect of meal (data not shown). However, a significant effect of time on both serum total cholesterol (P = 0.01) and HDL cholesterol (P < 0.0001) was observed.

ANOVA showed that meal had no significant effect on the AUC for total cholesterol when adjusted for the baseline concentration of total cholesterol or on the AUC for HDL cholesterol when adjusted for the baseline concentration of HDL cholesterol and the sequence of the test meals (data not shown).

Plasma concentrations of glucose, cholecystokinin, ghrelin, glucagon-like peptide 1, and peptide YY

The postprandial responses of glucose, CCK, ghrelin, GLP-1, PYY, and insulin are shown in Figure 2. Repeated-measures ANOVA showed a significant meal-by-time interaction on GLP-1 (P = 0.007) when adjusted for baseline concentration. Comparisons of adjusted least-squares means showed that the GLP-1 concentration was higher 60 min after intake of the Suppl
meal than after the LC \((P = 0.03)\), MC \((P = 0.01)\), and HC \((P = 0.005)\) meals. No significant differences were observed at the other time points, and no significant meal-by-time interaction or significant effect of meal was observed on CCK, ghrelin, PYY, glucose, or insulin when adjusted for baseline concentrations. However, a strong significant effect of time on CCK, ghrelin, PYY, and insulin \((P < 0.0001\) for all) was observed when adjusted for baseline concentration and on glucose \((P < 0.0001)\) when adjusted for baseline concentration and the sequence of the test meals.

The AUCs for CCK, GLP-1, ghrelin, PYY, glucose, and insulin were shown in Figure 2. ANCOVA showed that meal had no significant effect on the AUC for CCK, GLP-1, ghrelin, PYY, or insulin when adjusted for baseline concentrations or on the AUC for glucose when adjusted for the sequence of the test meals.

Palatability of the test meal

ANOVA showed that meal had a significant effect on the palatability of the test meal with regard to taste \((P = 0.02)\), visual appeal \((P < 0.0001)\), and overall palatability \((P < 0.0001)\) (data not shown). The subjects found that the taste of the HC meal was significantly better than the taste of the LC \((P = 0.0008)\) and the supplement \((P = 0.007)\) meals when adjusted for the sequence of the test meals. The subjects found that the HC meal was significantly more visually appealing than were the LC \((P < 0.0001)\) and Suppl \((P = 0.02)\) meals, and the LC meal was significantly less appealing than were the MC \((P = 0.0003)\) and Suppl \((P = 0.02)\) meals. The subjects found that the overall palatability of the HC meal was significantly better than that of the LC \((P < 0.0001)\) and the Suppl \((P = 0.0002)\) meals and that of the MC meal was significantly better than that of the LC \((P = 0.0003)\) and the Suppl \((P = 0.01)\) meals.

Appetite sensation

The postprandial responses in subjective appetite sensation measured by VAS are shown in Figure 3 (data on well-being and desire to eat meat or fish or something salty or sweet are not shown). Repeated-measures ANOVA showed no significant meal-by-time interaction and no significant effect of meal on hunger, satiation, fullness, prospective food consumption, thirst, well-being, or desire to eat meat or fish or something sweet, salty, or fatty after adjustment for sensation measured at baseline. However, a strong significant effect of time on all measures, except well-being, was observed \((P < 0.0001\) for all). ANCOVA showed that meal had no significant effect on AUC for any of the measures after adjustment for sensation measured at baseline (Figure 3).

At the end of the day, the subjects were served an ad libitum meal. ANOVA showed that meal did not significantly affect energy intake at this meal (data not shown). Meal had no significant effect on the palatability of the ad libitum meal, except on the smell and the visual appeal of the meal. The subjects found the smell of the ad libitum meal to be significantly better after intake...
of the HC ($P = 0.0008$) and the Suppl ($P = 0.04$) meals than after the intake of the LC meal. The subjects found the ad libitum meal to be significantly more visually appealing after intake of the HC ($P = 0.009$) and MC ($P = 0.01$) meals than after intake of the LC meal.

**DISCUSSION**

The present study evaluated the effects of calcium intake on postprandial fat metabolism as a proxy for fat absorption. The major finding was that a high calcium intake from dairy products, milk, and low-fat yogurt, but not from a calcium supplement, decreased postprandial lipidemia. No significant effect of a high calcium intake from either dairy products or the supplement on appetite sensations, appetite hormones, or calorie intake at the subsequent meal was found.

**Postprandial fat metabolism**

Consumption of the high-fat meals resulted, as expected, in pronounced postprandial lipidemia. Compared with the LC meal, the lipid response in chylomicron triacylglycerols was reduced by $\approx 17\%$ by the MC meal and by $\approx 19\%$ by the HC meal, which indicated that a high calcium intake from dairy products reduces the lipid response but also that a plateau value exists above which an increased calcium intake does not seem to have any additional effect. The total lipid response in plasma total triacylglycerols was not affected by calcium intake.

The decrease in the lipid response in chylomicron triacylglycerols may reflect either a decrease in fat absorption or an increase in chylomicron clearance. However, no evidence in the literature suggests that calcium intake interferes with chylomicron clearance, and the unaffected concentrations of total cholesterol and HDL cholesterol suggest that this is unlikely. It is therefore most likely that the decreased postprandial lipid response was due to decreases in fat absorption. It was previously shown that the lipid response in chylomicron triacylglycerols increases with the amount of fat ingested (0-50 g per meal) (34). As far as we know, the present study is the first to show that an increased calcium intake from dairy products exerts a lowering effect on...
postprandial fat absorption. However, several studies, in both animals and humans, have shown that calcium intake increases the fecal excretion of fat, presumably via the formation of insoluble calcium fatty acid soaps in the gut or by binding of bile acids, which impairs the formation of micelles (17–21, 24, 25, 35, 36).

Our main aim in studying postprandial fat metabolism was to examine whether the previously reported increased fecal fat excretion induced by a diet high in dairy calcium could be reflected in a diminished postprandial plasma excursion of triacylglycerol and chylomicron triacylglycerol, but we are aware that the findings might also have implications for the role of a diet high in dairy products in the risk of cardiovascular diseases (CVD). Observational studies have previously shown an inverse association between dairy and calcium intakes and CVD and dyslipidemia (2, 37–39). Orlistat, a gastrointestinal lipase inhibitor that reduces dietary fat absorption by ~30%, has been shown to have a positive effect on fasting lipid profile by decreasing fasting total cholesterol and LDL (40, 41) and reducing postprandial lipids (42). Although the effect of calcium in the present study is not as pronounced as the effect of Orlistat, it is likely that a long-term high intake of calcium from dairy products may have beneficial effects on the lipid profile and, thereby, on the risk of CVD. A beneficial effect on the lipid profile was previously shown in intervention studies using calcium from supplements or fortified foods (43, 44).

It was previously shown that calcium from dairy products has a more profound effect on body weight than does calcium from supplements (6, 14). The mechanism behind this difference is unknown. We previously suggested that this may be due in part to the time at which the supplement is consumed. If calcium is to inhibit the absorption of fat it, is a condition that fat and calcium are present in the gut at the same time (5). We therefore gave the supplement as a part of the meal and in a solution that mimicked the milk given with the other meals. However, the lipid response in chylomicron triacylglycerols was not significantly different after the intake of the LC and Suppl meals, which indicated that there must be another property of milk calcium that we did not take into account, eg, the chemical form of calcium, other bioactive components in dairy products, differences in the solubility...
of calcium from milk and calcium carbonate, or differences in pH. In dairy products, calcium is largely found as calcium phosphate and it is possible that phosphate contributes to the effect of calcium. It was previously shown that supplemental calcium increases the fecal excretion of phosphate and inhibits its absorption, probably because of the formation of insoluble calcium phosphate, which has been suggested to bind bile acids and thereby partly impairs the formation of micelles (25).

**Appetite sensation and regulation**

One of our considerations was that a partial inhibition of fat absorption by increased calcium intake could have a compensatory stimulatory effect on food intake, which might lead to increased food intake. However, we found no effect of a high calcium intake, from either dairy products or the supplement, on the sensation of appetite, on the secretion of the appetite-regulating hormones, or on energy intake at the subsequent ad libitum meal. A significant interaction was found between meal and time for GLP-1 secretion, but this was due to an inexplicably high concentration of GLP-1 60 min after intake of the supplement meal and it was not a persistent difference between the meals. In addition, there was no significant difference in the total GLP-1 response; therefore, we do not attach much importance to the significant interaction between meal and time. Our results indicate that the apparent inhibition of fat absorption does not have a stimulating effect on energy intake and that the previously observed effect of calcium on body weight and composition is not due to a reduced energy intake. However, we cannot exclude a subchronic effect of calcium on appetite.

We used a VAS to measure appetite sensation. The reproducibility and validity of the VAS was previously examined and found to be reliable (45). Flint et al found that, to detect a difference of 10% in the mean value, a study with a paired design found to be reliable (45). Flint et al found that, to detect a difference of 10% in the mean value, a study with a paired design found to be reliable (45). However, we found no effect of a high calcium intake, from either dairy products or the supplement, on the sensation of appetite, on the secretion of the appetite-regulating hormones, or on energy intake at the subsequent ad libitum meal. A significant interaction was found between meal and time for GLP-1 secretion, but this was due to an inexplicably high concentration of GLP-1 60 min after intake of the supplement meal and it was not a persistent difference between the meals. In addition, there was no significant difference in the total GLP-1 response; therefore, we do not attach much importance to the significant interaction between meal and time. Our results indicate that the apparent inhibition of fat absorption does not have a stimulating effect on energy intake and that the previously observed effect of calcium on body weight and composition is not due to a reduced energy intake. However, we cannot exclude a subchronic effect of calcium on appetite.

We used a VAS to measure appetite sensation. The reproducibility and validity of the VAS was previously examined and found to be reliable (45). Flint et al found that, to detect a difference of 10% in the mean value, a study with a paired design should include ≥18 subjects (20 subjects for the variable “desire to eat something salty”) to obtain a power of 0.9 (45). In the present study we used the AUC and not mean values in the data analyses. However, because the study included 18 subjects, it seems unlikely that the negative outcome found in the present study was due to type 2 errors. If possible, a gap should be entered because the study limitations apply to the entire study and not just the part dealing with appetite sensation and regulation.

The study had some limitations. Although all efforts were exerted to make the 4 meals as similar as possible, except for the calcium intakes, there were some differences in the compositions of the meals, and we could not rule out that these differences may have confounded the outcome. Moreover, we cannot exclude the possibility that an adaptation to a long-term high calcium intake may occur, which could reduce the effect. However, because our subjects had a relatively high habitual calcium intake, it seems unlikely that a total adaptation takes place. Further studies are necessary to examine this aspect further.

Consistent with our previous finding that a diet high in dairy calcium induces fat malabsorption in humans, the present findings indicate that the effect is also reflected in an attenuated postprandial lipid response after intake of dairy calcium from milk and low-fat yogurt, but not after intake of supplementary calcium. This may have been due to differences in the chemical form or to cofactors in the dairy products. The findings of the present study do not suggest any effect on appetite regulation as assessed by appetite sensations, appetite hormones, or ad libitum intake of a subsequent meal.

We are grateful to the laboratory and kitchen staff of the Department of Human Nutrition, especially Kirsten B Rasmussen, Charlotte Kostecki, and Yvonne Rasmussen, for their assistance. JKL, IT, and AA designed the study. JKL and SN collected the data. JH and JFR analyzed most of the hormones. JKL analyzed the data. All authors participated in the discussion of the results and commented on the manuscript. AA is a member of the Arla Nutrition Advisory Board and receives an honorarium for attending each board meeting.

**REFERENCES**


Relation of nutrients and hormones in polycystic ovary syndrome1–3

Sidika E Kasim-Karakas, Wendy M Cunningham, and Alex Tsodikov

ABSTRACT

Background: Insulin resistance, infertility, and hirsutism, common characteristics of polycystic ovary syndrome (PCOS), improve with even modest weight loss. Optimal dietary treatment for PCOS is not known.

Objective: We compared the effects of acute protein administration with those of glucose challenges on hormones related to obesity and insulin resistance (ie, cortisol and insulin), hirsutism [ie, dehydroepiandosterone (DHEA) and androstenedione], and hunger (ie, ghrelin).

Design: Patients with PCOS (n = 28; aged 26 ± 2 y) were tested with a 5-h oral-glucose-tolerance test (OGTT) and a euvolemic, euenergetic protein challenge.

Results: Glucose ingestion caused larger fluctuations in blood glucose and more hyperinsulinemia than did protein (P < 0.01, overall treatment-by-time interaction). During the protein challenge, cortisol and DHEA declined over 5 h. During OGTT, cortisol and DHEA increased after the third hour and began to show significant divergence from protein from the fourth hour (P ≤ 0.01). During OGTT, 18 patients who had a blood glucose nadir of <69 mg/dL had elevated cortisol (baseline: 10.4 ± 0.4; nadir: 5.9 ± 0.1; peak: 12.7 ± 0.9 μg/dL) and DHEA (baseline: 15.6 ± 1.3; nadir: 11.2 ± 1.0; peak: 24.6 ± 1.6 ng/mL) (P < 0.01), whereas the remaining 10 patients with a glucose nadir of 76 ± 2 mg/dL had no increase in adrenal steroids. Both glucose and protein suppressed ghrelin (from 935 ± 57 to 777 ± 51 pg/mL and from 948 ± 60 to 816 ± 61 pg/mL, respectively). After glucose ingestion, ghrelin returned to baseline by 4 h and increased to 1094 ± 135 pg/mL at 5 h. After the protein challenge, ghrelin remained below the baseline (872 ± 60 pg/mL) even at 5 h. The overall treatment effect was highly significant (P < 0.0001).

Conclusions: Glucose ingestion caused significantly more hyperinsulinemia than did protein, and it stimulated cortisol and DHEA. Protein intake suppressed ghrelin significantly longer than did glucose, which suggested a prolonged satiogenic effect. These findings provide mechanistic support for increasing protein intake and restricting the simple sugar intake in a PCOS diet. Am J Clin Nutr 2007;85:688–94.

KEY WORDS Polycystic ovary syndrome, PCOS, whey protein, adrenal steroids, ghrelin

INTRODUCTION

Polycystic ovary syndrome (PCOS) affects 6% of women; in the United States, ~6.8 million women have PCOS. The cardinal features of PCOS are androgen excess, ovarian dysfunction, and infertility (1). Most patients with PCOS are obese and insulin resistant; almost 50% of them meet the criteria of the metabolic syndrome (2, 3) as defined by the National Cholesterol Education Program Adult Treatment Panel III (4). Their risk of type 2 diabetes is significantly increased (1). Gestational diabetes may also be more common in PCOS (5–7), although the evidence is not conclusive (8).

It is important to recognize that even modest amounts of weight loss improve all of the manifestations of PCOS: weight loss decreases insulin resistance, serum androgen concentrations, ovarian size, and the number of ovarian cysts; it increases ovulation and fertility; and it improves the concentrations of plasma lipids (9–11). Despite the importance of weight loss, the optimal dietary treatment for PCOS is not known. High-protein diets are being promoted (12) because of their beneficial effects on satiety (13, 14), lean body mass (15–17), weight maintenance (18, 19), and lipid markers (10). High-fat diets are being used to reduce insulin response (20).

These desirable outcomes may partly be due to concomitant decreases in dietary carbohydrates, glycemic index, and glycemic load (21–23). Unfortunately, the mechanisms underlying the differential effects of nutrients are not known. Thus, the overall aim of this research was to compare the acute hormonal effects of eucaloric, euvolemic glucose with those of protein ingestion, with a long-term goal of defining the optimal dietary treatment strategies for persons with PCOS. We focused on the hormones influencing clinical features of PCOS. These hormones included insulin, adrenal steroids, cortisol—which causes insulin resistance, dyslipidemia, central obesity, and hypertension (24)—and dehydroepiandosterone (DHEA) and androstenedione—which constitute the substrates for peripheral testosterone synthesis (25). The hunger signal ghrelin was

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2 Supported by the California Dairy Research Foundation, the ALSAM Foundation, and the UC Davis General Clinical Research Center (biostatistical resource grant no. M01 RR19975). The whey protein was a gift from Glanbia Foods (Twin Falls, ID).

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measured to compare the potential effects of glucose with those of protein on satiety (26, 27).

Changes in these hormones were compared by using the oral-glucose-tolerance test (OGTT) and a eucaloric, euvolemic whey protein challenge. OGTT was used because it is well standardized to identify insulin resistance, impaired glucose tolerance, and diabetes (28). Whey protein was used because it is considered a “fast protein” that does not coagulate in the stomach and that rapidly increases plasma concentrations of amino acids, exerts an insulinotropic effect, and reduces postprandial glycemia in healthy subjects and persons with type 2 diabetes (13, 29).

Although standard OGTT is conducted over 2 h, this test was extended to 5 h because, in a previous study in patients with PCOS, we observed that several participants experienced symptoms suggestive of hypoglycemia after a 2-h OGTT (30). Therefore, a 5-h OGTT was used to document postprandial hypoglycemia, if it occurred (31).

The 5-h testing also allowed us to determine the changes in ghrelin during a period that is similar to the usual interval between meals. Previously published reports showed changes in ghrelin in 2–3 h, which is a shorter time than the usual between-meal interval (32–34).

SUBJECTS AND METHODS

Subjects

Twenty-eight patients with PCOS aged 18–45 y and with a body mass index (in kg/m²) of 25–40 were recruited. All participants were examined by the principal investigator (SEK-K), who is the director of the PCOS program at the Medical Center of the University of California, Davis. The participants fulfilled the Rotterdam criteria for PCOS (35) by having ovarian dysfunction, as evidenced by amenorrhea (no periods for >6 mo) or oligomenorrhea (<8 periods/y, cycle length >45 d, or both), and clinical (hirsutism) or laboratory evidence for hyperandrogenemia (total testosterone >54 ng/dL or free testosterone >9.2 pg/mL). Ultrasound evaluation of the ovarian structure was not carried out because most ultrasound reports do not provide the detailed information required by the Rotterdam criteria (presence of ≥12 follicles in each ovary, with each follicle measuring 2–9 mm in diameter; increased ovarian volume > 10 mL; or a combination, although the subjective appearance of PCO is not adequate).

Adult-onset 21-hydroxylase deficiency was ruled out by measuring basal concentrations of 17-hydroxyprogesterone in the morning. Although this measurement is routinely made during the follicular phase of the cycle, most patients had oligomenorrhea or amenorrhea, and, thus, the follicular phase could not be defined. The mean basal concentration of 17-hydroxyprogesterone was 53.0 ± 3.4 ng/dL; the highest value was 106.0 ng/dL. Because all of the basal morning concentrations of 17-hydroxyprogesterone were <200 ng/dL, none of the participants required a cortisyn-stimulation test. Prolactinoma was ruled out by measuring the serum prolactin concentration, and androgen-secreting tumors were ruled out based on serum testosterone (36–38). Cushing’s disease was ruled out clinically because the Rotterdam criteria require biochemical testing only when there is clinical suspicion. Patients were excluded if they used oral contraceptives, antiandrogen medications, insulin sensitizers, d-chiro inositol, or any other medications or supplements that affect weight or insulin sensitivity during the preceding 2 mo; have impaired glucose tolerance, diabetes mellitus, untreated hypothyroidism, and any other systemic illness such as renal, hepatic, and gastrointestinal disease; smoke; or drink > 2 alcoholic drinks/wk.

Written informed consent was obtained from all subjects. The study protocol was approved by the Human Subjects Committee of the University of California, Davis.

Oral-glucose-tolerance and protein challenge tests

The OGTT and protein challenge test studies were performed at the General Clinical Research Center of the University of California, Davis. The subjects were following their habitual diets and were weight stable. Their average carbohydrate intake was 255 g/d before testing. The studies were initiated between 0700 and 0800, after an overnight fast. An intravenous catheter was placed in the forearm. The OGTT and the protein challenge test were performed 7–10 d apart, in random order. For the OGTT, the participants ingested 75 g glucose (Glucola; Alliance Healthcare Corp, McGaw Park, IL) at 0 min. For the protein challenge, they ingested 75 g 98% pure, intact whey protein isolate containing no carbohydrate (Glanbia Foods, Twin Falls, ID). The glucose and the protein drinks were euvolemic and eucaloric. The blood samples were obtained at baseline and every 30 min thereafter for 5 h. The subjects remained supine in bed throughout the testing to avoid confounding effects of physical activity on blood glucose. The samples for glucose were collected in sodium fluoride–containing tubes on ice. Other samples were collected in either serum separation tubes or tubes containing EDTA or heparin. The nursing staffers who collected the samples and the laboratory personnel who carried out the assays were blinded. Before data analysis, a glucose concentration <69 mg/dL was defined as hypoglycemia.

Laboratory assays

Glucose was measured with the use of the hexokinase method in a clinical chemistry analyzer (Poly-Chem System, Cortlandt Manor, NY). Insulin and total ghrelin concentrations were measured with the use of radioimmunoassay kits (Linco Research Inc, St Charles, MO); the CVs were 8.2% and 7%, respectively. Cortisol, DHEA, and androstenedione were measured with the use of radioimmunoassay kits (Diagnostic System Laboratories, Webster, TX); the CVs were 5.3%, 7.8%, and 4.3%, respectively.

Statistical analysis

Statistical analysis was programmed with the R 2.1.1 language and environment (The R Foundation for Statistical Computing, Auckland University, Auckland, New Zealand; Internet: http://www.r-project.org/). Data were presented as means ± SEs, and P < 0.05 was considered significant. Linear mixed models with random intercept were used as a primary tool for the statistical analysis. The random intercept was shared by measurement on the same subject to take into account the subject-specific effect and to adjust for correlated error structure of observations. Time was treated as a categorical factor to allow enough flexibility in reproducing the complex longitudinal patterns observed in marker measurements. Interaction terms of type of challenge (glucose compared with protein) with time were introduced to address the effect at each particular time point as well as overall. Symbolically, the model can be represented in the form of Marker ~ intercept + main effect (time) + interaction effect (time × treatment)/subject, where intercept models mean marker
value at time zero before treatment is applied. Main effect (time) models the mean marker response over time under the OGTT challenge. Interaction effect (time \times treatment) models the difference in mean marker response by time point between protein challenge and OGTT. All terms are adjusted for the subject-specific effect that contributes to between-subject variability of measurements and a correlation of measurements over time. Validity of the model was assessed with the use of residual plots. Testing for treatment differences was done for every time point as well as overall; the latter testing involved testing all coefficients in the treatment by block of time. A model-based analysis that is resistant to type I error inflation resulting from multiple comparisons was used. All model-based hypothesis testing was based on the so-called Wald test, which is equivalent to the likelihood ratio test for large samples. Akaike information criterion was monitored to prevent inflated type I error and overfitting.

As a descriptive analysis, the area under the curve (AUC) summary measure was computed for each subject. A paired t test was used to assess the treatment effect on the AUC. AUC summary measures were computed with the use of the Sympon quadrature method. In numeric computation of the AUC integrals, missing values were treated according to “last observation carried forward” principle. AUC analysis was performed for descriptive and interpretation purposes, and no adjustment was made for multicomplications.

Baseline, lowest, and peak values for cortisol and DHEA were defined on the basis of the longitudinal trajectory of the marker in each subject. The baseline was the value obtained at 0 min before the administration of glucose or protein challenge; the nadir was the lowest value; the peak was the highest value after the nadir. These definitions were adopted before (and without the knowledge of the results of) the statistical analysis. A statistical linear mixed model was then applied to such clinically assessed measurements.

RESULTS

Clinical characteristics of the participants

The mean age was 26 ± 2 y, the weight was 97.5 ± 4.1 kg, and the body mass index was 35.9 ± 1.2. The mean serum concentrations were 0.84 ± 0.12 ng/mL for testosterone, 37.2 ± 6.5 nmol/L for sex hormone–binding globulin, and 215 ± 32 ng/mL for DHEA sulfate (DHEAS). (The reference values in 19 women with regular menstrual cycles were 0.27 ± 0.03 ng/mL for testosterone, 68.5 ± 6.6 mmol/L for sex hormone–binding globulin; and 116 ± 24 ng/mL for DHEAS.) The mean morning 17-hydroxyprogesterone concentration was 53.0 ± 3.4 ng/dL, and the highest value was 106.0 ng/dL.

Changes in plasma glucose and insulin

Twenty-eight subjects completed the 5-h OGTT, and 23 subjects completed both the 5-h OGTT and the protein challenge test (Figure 1). The order of the tests was randomized, and no effect of the treatment order on test results was observed. Protein challenge did not significantly affect plasma glucose (baseline: 97 ± 2 mg/dL; peak at 30 min: 103 ± 3 mg/dL; nadir at 240 min: 91 ± 2 mg/dL; $P = 0.30–0.75$ when glucose concentrations at different time points were compared with the baseline concentration). During the OGTT, plasma glucose concentrations were higher during the first 3 h but below the baseline afterward (baseline: 98 ± 2 mg/dL; peak at 60 min: 156 ± 8 mg/dL; nadir at 240 min: 76 ± 2 mg/dL). Except $t = 0$ (no treatment at this point) and $t = 180$ min (crossing point), a significant treatment effect was observed at all time points ($P < 0.0001$). Although whey protein also increased insulin secretion (as is consistent with the known stimulatory effects of amino acids), plasma insulin concentrations were significantly higher after glucose ingestion. For example, the peak insulin concentrations were 132.1 ± 12.5 μU/mL at 30 min after glucose ingestion and 97.2 ± 12.2 μU/mL at 60 min after protein ($P < 0.001$). Overall, a highly significant difference was observed in insulin concentrations by treatment ($P < 0.0001$, model-based). However, timepoint differences at 3 h and thereafter showed no significant difference. As seen in Figure 1, it was evident that the insulin response during the first 2.5 h was responsible for the overall significance, when glucose concentrations were above the baseline; the glucose group had significantly higher insulin concentrations than did the protein group. Because of the crossing curves, overall comparison of insulin AUC showed no significant difference: some of the

![Figure 1](image-url)
effects before and after 3 h canceled each other out. Yet, when only the early part of the curve is included in the AUC analysis, the result was highly significant (P = 0.009, paired t test).

Changes in serum cortisol, DHEA, and androstenedione

During the protein challenge test, serum cortisol and DHEA declined during 5 h (Figure 2). Cortisol decreased from a baseline of 10.1 ± 0.6 to 6.2 ± 0.4 μg/dL, and DHEA decreased from a baseline of 15.6 ± 1.6 to 10.7 ± 1.0 ng/mL (P < 0.0001). During the OGTT, serum cortisol and DHEA declined until the third hour but did not decrease further after that time. Cortisol decreased from a baseline of 10.2 ± 0.6 to 7.6 ± 0.5 μg/dL at the third hour and then increased to 8.9 ± 0.5 μg/dL at 5 h; DHEA decreased from a baseline of 15.4 ± 1.5 to 11.7 ± 1.1 ng/mL at the third hour and then increased to 15.7 ± 1.4 ng/mL. Timepoint model–based analysis showed a highly significant difference by treatment at or after t = 240 min for cortisol (P < 0.01) and DHEA (P < 0.001). This observation is confirmed by a paired t test comparing AUC by treatment for cortisol (P = 0.090) and DHEA (P = 0.004). The differences in the AUC were smaller because the values responsible for these differences were concentrated at the late portion of the experiment, which explained the borderline result for cortisol AUC.

Changes in androstenedione also showed similar trends. During the OGTT, the average androstenedione values were 1.77 ± 0.20, 1.46 ± 0.10, 1.39 ± 0.13, 1.56 ± 0.11, 1.58 ± 0.12, and 1.64 ± 0.10 ng/mL at 0 min and 1, 2, 3, 4, and 5 h, respectively. During the protein challenge test, these values were 1.53 ± 0.16, 1.23 ± 0.10, 1.18 ± 0.14, 1.24 ± 0.13, 1.32 ± 0.15, and 1.40 ± 0.13 ng/mL, respectively. The protein challenge showed consistently lower concentrations of androstenedione than did OGTT. However, the overall treatment effect was not significant (P = 0.221, model-based).

Next, we examined whether the increases in adrenal steroids were related to the changes in plasma glucose. Before data analysis, we defined the hypoglycemic group as subjects with a glucose nadir of <69 mg/dL during OGTT. We contrasted baseline and peak measurements against the nadir and introduced an interaction term to allow testing for the peak response. The hypoglycemic group (n = 18) had larger increases in cortisol and DHEA than did the nonhypoglycemic group (n = 10). Cortisol concentrations were 10.1 ± 0.5 μg/dL at baseline, 5.9 ± 0.1 μg/dL at nadir, and 12.7 ± 0.9 μg/dL at peak in the hypoglycemic group and 10.4 ± 0.4 μg/dL at baseline, 6.1 ± 0.1 μg/dL at nadir, and 7.8 ± 1.0 μg/dL at peak in the nonhypoglycemic group (P < 0.01). The DHEA concentrations were 15.6 ± 1.3 ng/mL at baseline, 11.2 ± 1.0 ng/mL at nadir, and 24.6 ± 1.6 ng/mL at peak in the hypoglycemic group and 13.2 ± 3.3 ng/mL at baseline, 9.1 ± 1.2 ng/mL at nadir, and 11.9 ± 1.9 ng/mL at peak in the nonhypoglycemic group (P < 0.01). The hypoglycemic group also had a greater increase in androstenedione from the nadir to the peak than did the nonhypoglycemic group (P = 0.06).

Characteristics of the hypoglycemic group were that these 18 subjects were significantly less obese (94.1 ± 5.0 kg) and had significantly lower fasting plasma glucose (91 ± 2 mg/dL) than did the remaining 10 subjects (weight: 106.8 ± 4.1 kg, P = 0.07; fasting glucose 106 ± 4 mg/dL, P < 0.001). No difference was observed between fasting insulin concentrations in the 2 groups.

Changes in plasma ghrelin

Both protein and glucose ingestions suppressed ghrelin (from 935 ± 57 to 777 ± 51 pg/mL and from 948 ± 60 to 816 ± 61 pg/mL, respectively). After glucose ingestion, ghrelin returned to baseline by 4 h and increased to 1094 ± 135 pg/mL at 5 h. After the protein challenge, ghrelin remained below the baseline even at 5 h (872 ± 60 pg/mL) (Figure 3). Significant treatment differences were found during the 3–5-h period, and the overall treatment effect was highly significant (P < 0.0001). The ghrelin response during OGTT was not related to the presence or absence of hypoglycemia (data not shown).

DISCUSSION

This study showed that glucose ingestion caused larger fluctuations in blood glucose and more hyperinsulinemia than did the intact whey protein. Hyperinsulinemia contributes to obesity by stimulating lipoprotein lipase and fatty acid synthase. The lipoprotein lipase enzyme releases the fatty acids from the triacylglycerol-rich lipoproteins, and fatty acid synthase facilitates the storage of fatty acids as triacylglycerol in the adipose tissue. Simultaneously, insulin inhibits the hormone-sensitive lipase, thus interfering with the mobilization of the triacylglycerols stored in the adipose tissue. Hyperinsulinemia also contributes to the other endocrine abnormalities seen in PCOS: insulin...
concentrations and DHEA occurred in all the subjects who had blood glucose injection of insulin to induce an adequate stress response. The are stored in the intraabdominal fat depots, resulting in central

panied by hyperinsulinemia, as seen in PCOS, these fatty acids concentration of free fatty acids in the circulation. When accom-

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increased counterregulatory hormones during asymptomatic hy-

diabetes and the report of Solter et al (43, 44) that showed in-

increased counterregulatory hormones during asymptomatic hy-

FIGURE 3. Mean ± SE changes in total plasma ghrelin during oral-glucose-tolerance test (— — ; n = 28) and protein challenge (— — ; n = 23). Differences between responses to the 2 treatments are expressed through treatment-by-time interaction effect. Overall, the interaction effect is highly significant (P < 0.001). Key timepoint P values are shown on the graph. All tests are based on the Wald test performed by using a linear mixed model applied to all available data.

increases ovarian androgen production by stimulating cyto-

chrome P450c17α activity (39) and adrenal steroidogenesis in

response to adrenocorticotropic (ACTH) (40). Insulin also aug-

ments pituitary release of the luteinizing hormone (41). Thus, protein that causes less hyperinsulinemia may be a more prefer-

able nutrient than glucose for patients with PCOS, although this possibility remains to be further tested by the comparison of protein-rich and carbohydrate-rich whole foods.

After ingesting glucose, two-thirds of the patients with PCOS had postprandial hypoglycemia and greater cortisol and DHEA secretion. Although the protein challenge delivered an identical amount of calories and raised plasma insulin, it did not alter plasma glucose or stimulate adrenal steroids. It is well established that hypoglycemia stimulates secretion of pituitary ACTH, which, in turn, stimulates adrenal steroids (31). In fact, hypoglycemia is used as endocrine testing to assess the integrity of the hypothalamic-pituitary-adrenal axis. During this test, the blood glucose concentration is lowered to <50 mg/dL by an injection of insulin to induce an adequate stress response. The novel finding of our study was that the prompt increase in cortisol and DHEA occurred in all the subjects who had blood glucose concentrations < 69 mg/dL during OGTT. This observation was similar to that of the report of Spyer et al (42) that showed increased counterregulatory hormone secretion with plasma glucose concentrations < 67 mg/dL in patients with well-controlled diabetes and the report of Solter et al (43, 44) that showed increased counterregulatory hormones during asymptomatic hy-

goglycemia in obese subjects.

The increase in serum cortisol can contribute to the progress-
on of obesity, insulin resistance, and metabolic syndrome. Cor-

tisol contributes to obesity by increasing caloric intake and pref-

erential consumption of sweet and fatty foods (45, 46). Cortisol increases lipolysis in the adipose tissue and thereby raises the concentration of free fatty acids in the circulation. When accom-

panied by hyperinsulinemia, as seen in PCOS, these fatty acids are stored in the intraabdominal fat depots, resulting in central

obesity (47), and are used for triacylglycerol production in the liver, contributing to hyperlipidemia (48). Cortisol increases glu-
cose production by stimulating gluconeogenic enzymes in the liver (49), and it causes insulin resistance in the muscle. In fact, effects of cortisol in the muscle may be directly related to the development of metabolic syndrome (50). Because ~60% of patients with PCOS are insulin resistant (51) and 46% of them manifest the metabolic syndrome (3), nutrient-related postpran-
dial cortisol secretion may have significant consequences in PCOS.

We also observed increases in adrenal androgens during OGTT. The increase seen in DHEA was larger than the increase in androstenodione. Similarly, Farah-Eways et al (52) reported that, after ACTH stimulation, serum DHEA increased by 222% and androstenodione increased by 31% in patients with PCOS, whereas DHEA increased by 266% and androstenodione increased by 68% in healthy control women. Although testosterone is the main excessive androgen in PCOS, depending on patients’ ethnicity, 20–30% of patients with PCOS have high serum concentra-
tions of DHEAS (25, 53). Brothers and sisters of the pa-

tients with PCOS also have higher plasma DHEAS concentra-
tions (54, 55). Thus, adrenals can be an important source of androgens. Although the main adrenal androgens, DHEA and androstenodione, have minimal direct biological effects, they constitute the primary substrates for testosterone synthesis in the peripheral tissues. In PCOS patients, DHEA secretion in re-
sponse to ACTH (56) and the conversion of adrenal androgens to testosterone by 5α-reductase (57) are increased. It is not yet known whether nutrient-related postprandial secretion of ad-

renal androgen can contribute to the hyperandrogenemia in PCOS. Because testosterone has a long half-life, our study was not designed to address this possibility, and a single episode of a rise in adrenal steroids occurring during the last 2 h of the OGTT cannot be expected to influence plasma testosterone concentrations.

Our observations related to ghrelin can be important to the nutritional management of PCOS. The findings that protein in-

gestion suppressed ghrelin for a longer time than glucose and that it did not cause a rebound increase are consistent with the recent report of Blom et al (58) and suggest that protein intake may prolong satiety. Previous reports indicated that protein intake neither suppressed nor increased ghrelin concentrations (32–34). Those studies compared the ingestion of glucose with that of solid mixed meals or determined the ghrelin response during 2-3 h. We compared glucose and protein in equal weights, volumes, and calories over a longer period. The protein-induced suppres-
sion of ghrelin was probably due to the increase in insulin. Insulin is known to suppress ghrelin independent of the changes in glu-
cose concentrations (59, 60). Broglia et al (26) showed that both oral glucose intake and intravenous insulin administration sup-

ressed ghrelin, although they had opposite effects on plasma glucose. It is interesting that the same study showed that intra-

venous arginine did not suppress ghrelin, despite increasing in-

sulin. Therefore, the oral route may be necessary for protein-

induced suppression of ghrelin. Alternatively, the intrinsic

properties of proteins may be important. The protein used in this study, whey protein, does not coagulate in the stomach, increases plasma concentrations of amino acids rapidly, and may suppress hunger more effectively (29).
In summary, oral glucose intake caused larger fluctuations in plasma glucose, increased hyperinsulinemia, and stimulated adrenal steroid secretion in patients with PCOS. In addition, glucose intake suppressed the hunger signal ghrelin for a shorter period of time than did protein intake. These acute challenge studies showed that nutrients have significantly different endocrine effects and that protein may be a preferred nutrient over glucose for patients with PCOS. The findings of these acute studies need to be validated with the use of natural foods and carbohydrate-enriched rather than protein-enriched diets. Further research is necessary to determine whether foods with high glycemic load contribute to the progression of obesity, insulin resistance, and hirsutism in PCOS patients by stimulating counterregulatory hormones and increasing hunger. In addition, these studies need to be extended to both nonobese and obese women without PCOS to determine whether postprandial hypoglycemia has significant adverse consequences in these populations.

We thank Nicole Harris for her diligent work as the study coordinator and Rogelio Almario and Nancy Fong for their work as research assistants. SEK designed the study and supervised all the clinical steps and laboratory assays; WMC helped to formulate the supplements; AT analyzed the data; SEK-K wrote the draft of the manuscript, and all authors contributed to the revision of the manuscript. None of the authors had a personal or financial conflict of interest.

REFERENCES

Comparison of nutritional and inflammatory markers in dialysis patients with reduced appetite\textsuperscript{1–3}

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ABSTRACT

Background: Anorexia is common in chronic kidney disease and worsens as the disease progresses. Sex hormones and inflammatory cytokines may be related to feeding behavior.

Objective: We hypothesized that appetite would be related to inflammation and outcome in hemodialysis patients but that sex may account for differences in the symptoms associated with poor appetite.

Design: A cross-sectional study was conducted in patients undergoing prevalent hemodialysis (n = 223; 127 M; \( \bar{x} \pm S D \) age: 66 ± 14 y). Anthropometric markers of body composition, handgrip strength, and nutritional and inflammatory status were measured, and 3 groups according to their self-reported appetite were established. Overall mortality was assessed after 19 mo (range: 2–29 mo) of follow-up.

Results: Poor appetite was associated with a longer vintage time, increased inflammation (higher serum concentrations of interleukin 6 and C-reactive protein), and a worse nutritional status (lower serum concentrations of insulin-like growth factor I, albumin, urea, and creatinine). However, across worsening appetite scale, handgrip strength was incrementally lower in men but not in women (multivariate analysis of variance). In a multivariate logistic regression analysis [odds ratio (OR): 0.41; 95% CI: 0.24, 0.72], appetite loss was associated with sex [odds ratio (OR): 0.41; 95% CI: 0.24, 0.72], insulin-like growth factor I (3.58; 2.10, 6.32), and C-reactive protein [OR: 0.41; 95% CI: 0.24, 0.72]. Appetite loss was associated with worse clinical outcome even after adjustment for age, sex, inflammation, dialysis vintage, and comorbidity (likelihood ratio = 44.3; \( P < 0.0001 \)).

Conclusions: These results show a close association among appetite, malnutrition, inflammation, and outcome in patients undergoing prevalent hemodialysis. Moreover, our data suggest that uremic men may be more susceptible than are women to inflammation-induced anorexia.  

KEY WORDS  Hemodialysis, inflammation, malnutrition, appetite, anorexia, sex, outcome

INTRODUCTION

Chronic kidney disease (CKD) is characterized by an exceptionally high mortality rate, primarily due to cardiovascular disease (CVD). Chronic inflammation is common among patients with renal disease and probably contributes to CVD (1). Moreover, the prevalence of protein energy wasting (PEW) among patients with CKD is high and is associated with a proinflammatory state (2, 3). Malnutrition, inflammation, and atherosclerosis often coexist among patients with CKD, and each of these risk factors independently predicts outcome in these patients (4, 5).

Patients with CKD frequently experience loss of appetite (anorexia), which increases in severity during the progression of the disease and which may lead to metabolic disturbances, PEW cachexia, and high rates of morbidity and mortality (6, 7). Multiple mechanisms may be involved in the pathophysiology of anorexia in CKD, and it is unclear how and to what extent a reduced appetite is a cause or a consequence of inflammation, PEW, or both. However, evidence is now convincing that inflammatory cytokines play an important role in the control of appetite, food intake, and energy homeostasis by interacting in several central nervous system pathways and that these cytokines also have both direct and indirect effects on specific brain areas (7–9). Indeed, Kalantar-Zadeh et al (10) found that inflammation was strongly associated with appetite and outcome in a well-characterized group of patients undergoing prevalent hemodialysis.

Sex is physiologically associated with feeding behavior (11) and inflammatory status (12), and men undergoing dialysis who have inflammation seem to have a shorter survival than do women with the same conditions (13). A sex-specific regulation of feeding is unclear; however, higher anorectic signals and earlier satiety were reported in men with chronic illnesses (14, 15).

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perhaps contributing to a different response pattern to anorexi-
genic diseases (eg, heart failure and cancer) among men and
women (16).

Recently, the Hemodialysis (17) and the Nutritional and In-
flammatory Evaluation of Dialysis (10) studies, with the use of a
simple, standardized questionnaire to determine a patient’s self-
reported appetite intensity, showed some consistent and some
contradictory results about the appetite evaluation in patients
undergoing hemodialysis. Hence, the current study strives to
elucidate some of these inconsistencies and hypothesizes that sex
might account for differences in the regulation of appetite and its
relation to inflammation and PEW. To test these hypotheses, we
performed a cohort study in a well-characterized group of pa-
tients undergoing prevalent hemodialysis to investigate the con-
ditions associated with loss of appetite and the predictive value of
self-reported appetite in relation to sex.

SUBJECTS AND METHODS

Subjects

The current study was performed at the Karolinska University
Hospital in Stockholm (including 4 satellite dialysis units), Dan-
deryds Hospital, and Uppsala Academic Hospital. It was a post
hoc analysis from a cross-sectional study with a follow-up that
originally aimed at investigating the variability of inflammatory
markers in patients undergoing prevalent hemodialysis (n = 254)
over time. Recruitment of the patients occurred from October
2003 through March 2004. All patients who were currently re-
ceiving regular therapy at any of the units were invited to par-
ticipate; 6 patients declined, and 1 patient with HIV infection was
excluded. The 247 eligible patients were then followed for 12 wk,
during which time the concentration of high-sensitivity
C-reactive protein (hs-CRP) was measured weekly. Because the
aim of the original study was to investigate the variability of
inflammatory markers, patients were excluded from the study if
available for fewer than 6 of these weekly hs-CRP measure-
ments. Eleven patients were excluded because insufficient base-
line clinical information was available, 7 were excluded because
of insufficient hs-CRP measurements, and 1 patient died. The
remaining 228 patients were further followed for assessment of
overall and cardiovascular mortality in relation to biochemical
markers. For the present study, 5 more patients were excluded
because no self-report of appetite intensity was available, which
left 223 patients for inclusion in the analysis. The current study
is limited only to baseline values. A patient’s flow chart is shown
in Figure 1.

Survival was determined after a mean follow-up of 19 mo
(range: 2-29 mo). Each patient’s medical chart was thoroughly
reviewed by a nephrologist (SS-J), who extracted data pertaining
to underlying kidney disease, history of CVD, and other comor-
bid conditions. The Davies comorbidity index was used to assess
the severity of comorbid conditions (18).

Written informed consent was obtained from each patient. The
study protocol was approved by the Ethics Committee of Karo-
linska Institute at Huddinge University Hospital (Stockholm,
Sweden).

Nutritional status and appetite assessment

Self-reported appetite is a part of the subjective global assess-
ment (SGA) questionnaire (2, 19). The SGA questionnaire in-
cludes 6 different components: 3 subjective assessments that are
performed by the patients and that concern the patient’s history
of weight loss, incidence of anorexia, and incidence of vomiting
and 3 assessments that are performed by the evaluators and that
are based on the subjective grading of muscle wasting, the pres-
ence of edema, and the loss of subcutaneous fat. On the basis of
these assessments, each patient received a nutritional status
score: 1) normal nutritional status, 2) mild malnutrition, 3) mod-
erate malnutrition, and 4) severe malnutrition. For the purposes
of the current study, malnutrition was defined as an SGA score
>1. With respect to the self-reported appetite assessment, all
patients were asked to grade their appetite according to the fol-
lowing scale: 1) good, 2) sometimes bad, 3) often bad, and 4) 
always bad.

Anthropometric evaluation

Body weight, body mass index (BMI; in kg/m²), and anthro-
pometric and dynamometric measurements were taken on a dia-
lysis day. Height was obtained from the patient’s chart. Fat mass
and lean body mass were assessed according to Durnin et al (20),
and body composition was assessed by using the 4 skinfold
thicknesses (biceps, triceps, subscapular, and suprailiac), mea-
sured with a conventional skinfold caliper (Cambridge Scientific
Instruments, Cambridge, MD). Fat BMI (FBMI) and lean BMI
were calculated (21). Midarm circumference was measured with
a plastic tape measurer and was normalized with measurements
from healthy subjects; midarm muscle circumference was cal-
culated by using the following formula: midarm circumference
– π × triceps skinfold thickness (20). Handgrip strength was
measured in both the dominant and nondominant hands by using
a Harpenden Handgrip Dynamometer (Yamar, Jackson, MI).
Each measurement was repeated 3 times for each arm, and the
highest value for each arm was noted. For our analysis, we used
the right arm handgrip strength, because fistulas were usually
placed in the left arm.

Laboratory analysis

Blood samples were collected before the dialysis session. The
plasma was separated within 30 min, and samples were kept
frozen at −70 °C if not analyzed immediately. Concentrations of
hs-CRP (nephelometry), serum urea, hemoglobin, hypochromic

FIGURE 1. Flow of participants during the study. hs-CRP, high-
sensitivity C-reactive protein.
red blood cells, serum creatinine, and serum albumin (bromocresol purple) were measured by routine methods at the Department of Laboratory Medicine, Karolinska University Hospital, Huddinge.

Plasma concentrations of insulin-like growth factor I (IGF-I) and interleukin 6 were measured in an Immulite Automatic Analyzer (Diagnostic Products Corporation, Los Angeles, CA). We used assays manufactured for this analyzer and followed the manufacturer’s instructions.

Statistical analysis

Normally distributed variables were expressed as means ± SDs (unless otherwise noted), and nonnormally distributed variables were expressed as medians and ranges. Statistical significance was set at $P < 0.05$. All statistical analyses were performed with SAS statistical software (version 9.1; SAS Institute Inc, Cary, NC). A 2-factor multivariate analysis of variance with Wilks $\lambda$ was used to measure the degree of correlation among the variables. The model included a test for the effect of order. The general linear model procedure with least-squares means was used to identify significant interactions among factors. When a significant interaction was found between factors ($A \times B$), those factors were identified with simple main-effects tests. Differences among the appetite groups were analyzed with the Kruskal-Wallis analysis of variance, followed by the post hoc Dunn test for nonparametric comparisons. A chi-square test was used for categorical variables. A multinomial logistic regression model was used to assess the predictors for self-reported appetite; this model included all variables significantly associated with self-reported appetite in univariate analysis. Survival analyses used the Kaplan-Meier survival curve and the Cox proportional hazards model [Figure 4B; likelihood ratio (chi-square test): 6.85; $P < 0.05$]. Significant differences in survival among the 3 groups persisted after the analysis was adjusted for potential confounding factors [ie, age, sex, inflammation (CRP > 10 mg/L), dialysis vintage, and Davies comorbidity index] by using a Cox proportional hazards model [Figure 4B; likelihood ratio (chi-square test): 44.3; $P < 0.0001$]. Self-reported poor appetite was significantly associated with higher mortality [hazard ratio (HR): 2.72; 95% CI: 1.29, 5.72; $P = 0.008$]. Although the patients reporting sometimes bad appetite also tended to be associated with higher mortality, this association was not significant (HR: 1.26; 95% CI: 0.63, 2.53).

RESULTS

The study population consisted of 223 patients undergoing hemodialysis (127 men; 57%) with a median age of 66 y (range: 23–87 y). The patients had undergone hemodialysis for a median of 30 mo (range: 1-378 mo) and had an average BMI of 24.5 ± 5.2. Of these patients, 57 (25%) had diabetes, 43 (19%) had CVD, 84 (38%) had inflammation (hs-CRP > 10 mg/L), and 96 (43%) were malnourished (SGA score $> 1$).

Self-reported appetite score was analyzed. Of the 223 patients, 124 (56%) reported a good appetite, 65 (29%) described their appetite as sometimes bad, 20 (9%) described it as often bad, and 14 (6%) described it as always bad. Because of the small sample size for the groups with poor appetite, the score was rearranged into 3 main groupings for further comparisons; according to these new categories, 124 patients (56%) reported a good appetite, 65 (29%) reported sometimes bad appetite, and 34 (15%) reported a poor appetite (combining often-bad and always-bad appetites).

Clinical data, anthropometric measurements, biochemical markers, and self-reported ratings of appetite for all 223 patients are shown in Table 1, along with the multivariate analysis of variance interactions. The proportions of men became progressively and significantly lower as the appetite scale worsened (64% men in the category good appetite; 54% men in the category sometimes bad appetite; 35% men in the category poor appetite; $P = 0.0008$). No significant differences were observed in the Davies comorbidity scores between patients in the 3 appetite categories.

A poor appetite was associated with a worse nutritional status, because serum concentrations of serum albumin, creatinine, and urea and IGF-I were lower among appetite categories. Furthermore, a poor appetite was also associated with worse anthropometrics, measured as BMI and FBMI, and with a higher inflammatory status, measured as serum concentrations of hs-CRP and interleukin 6 (Figure 2). Across the worsening appetite scale, handgrip strength was incrementally lower in men ($P = 0.0002$; Figure 3) but not in women.

To determine which variables were significantly associated with appetite, we created a multinomial logistic regression model (pseudo $r^2 = 0.19$; $P < 0.0001$) in which IGF-I ($\geq 159$ ng/mL compared with $< 159$ ng/mL; odds ratio (OR): 3.58; 95% CI: 2.10, 6.32), presence of inflammation (hs-CRP $< 10$ mg/L compared with $> 10$ mg/L; OR: 2.39; 95% CI: 1.34, 4.11), and sex (women compared with men; OR: 0.41; 95% CI: 0.24, 0.72) were significant predictors of appetite (Table 2).

Kaplan-Meier curves illustrating the cumulative proportion of surviving patients in different appetite groups are presented in Figure 4. As expected, the survival rate was worse for patients reporting a poor appetite than for patients reporting a good appetite [Figure 4A; log rank (chi-square test): 6.85; $P < 0.05$]. Significant differences in survival among the 3 groups persisted after the analysis was adjusted for potential confounding factors [ie, age, sex, inflammation (CRP > 10 mg/L), dialysis vintage, and Davies comorbidity index] by using a Cox proportional hazards model [Figure 4B; likelihood ratio (chi-square test): 44.3; $P < 0.0001$]. Self-reported poor appetite was significantly associated with higher mortality [hazard ratio (HR): 2.72; 95% CI: 1.29, 5.72; $P = 0.008$]. Although the patients reporting sometimes bad appetite also tended to be associated with higher mortality, this association was not significant (HR: 1.26; 95% CI: 0.63, 2.53).

DISCUSSION

This study shows a close relation among self-reported appetite, PEW, inflammation, and outcome for patients undergoing hemodialysis (10, 17). Moreover, this study suggests that sex may determine the severity of symptoms, such as handgrip strength, among patients who report a poor appetite. This observation supports the hypothesis that uremic men may be more susceptible than are women to inflammation-induced anorexia. A reduced appetite is an early and common sign of uremia and becomes increasingly more prominent as the glomerular filtration rate declines (22–24). In this study, we confirm previous findings (10, 17) about the predictive value of self-reported appetite on the outcome of hemodialysis patients, both men and women. This predictive value is mainly due to the expected correlation with PEW but also to a strong association with inflammation. In contrast to the findings by Kalantar-Zadeh et al (10), but in agreement with the findings of Burrowes et al (17), our findings indicate that loss of appetite is accompanied by poorer anthropometric values. In addition, we found a strong inverse association between appetite and plasma concentrations of IGF-I; these concentrations were suggested to be a good nutritional marker relating to lean body mass for patients undergoing dialysis (25). Because IGF-I is part of the major anabolic
TABLE 1
Clinical characteristics, anthropometric measurements, and biochemical markers of nutritional and inflammatory status in 223 prevalent hemodialysis patients, divided according to sex and self-reported appetite

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Men</th>
<th>Women</th>
<th>MANOVA&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Self-reported appetite scale</strong></td>
<td>Good (&lt;i&gt;n = 80&lt;/i&gt;)</td>
<td>Sometimes bad (&lt;i&gt;n = 35&lt;/i&gt;)</td>
<td>Poor (&lt;i&gt;n = 12&lt;/i&gt;)</td>
</tr>
<tr>
<td><strong>Men</strong> (&lt;i&gt;n = 127&lt;/i&gt;)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Women</strong> (&lt;i&gt;n = 96&lt;/i&gt;)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>64 (29–86)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>62 (37–85)</td>
<td>65 (51–84)</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>26</td>
<td>32</td>
<td>25</td>
</tr>
<tr>
<td>CVD (%)</td>
<td>18</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>Davies comorbidity score</td>
<td>Low</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>51</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>High (%)</td>
<td>29</td>
<td>26</td>
</tr>
<tr>
<td>Biochemical markers of nutritional and inflammatory status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>123 ± 12&lt;sup&gt;7&lt;/sup&gt;</td>
<td>116 ± 12</td>
<td>119 ± 12</td>
</tr>
<tr>
<td>Hypochromic RBCs (%)</td>
<td>1.8 ± 2.7</td>
<td>4.1 ± 3.9</td>
<td>5.3 ± 5.9</td>
</tr>
<tr>
<td>Serum albumin (g/L)</td>
<td>35.4 ± 3.6</td>
<td>34.2 ± 5.1</td>
<td>31.1 ± 4.6</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>885 ± 211</td>
<td>738 ± 208</td>
<td>732 ± 165</td>
</tr>
<tr>
<td>Serum urea (mmol/L)</td>
<td>25.6 ± 5.9</td>
<td>22.2 ± 5.8</td>
<td>22.5 ± 3.8</td>
</tr>
<tr>
<td>IGF-I (ng/mL)</td>
<td>191 (60–563)</td>
<td>137 (25–282)</td>
<td>90 (52–276)</td>
</tr>
<tr>
<td>hs-CRP (mg/L)</td>
<td>5.4 (0.4–151.0)</td>
<td>11.0 (0.6–98.0)</td>
<td>26.6 (3.6–123.0)</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>7.1 (1.7–83)</td>
<td>9.1 (1.6–91)</td>
<td>15.8 (4.6–65)</td>
</tr>
<tr>
<td>Anthropometric measurements</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>25.1 ± 4.1</td>
<td>22.7 ± 4.2</td>
<td>21.6 ± 3.8</td>
</tr>
<tr>
<td>LBMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>17.5 ± 2.3</td>
<td>16.4 ± 2.2</td>
<td>16.4 ± 2.5</td>
</tr>
<tr>
<td>FBMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>7.4 ± 2.3</td>
<td>6.1 ± 2.6</td>
<td>5.1 ± 1.7</td>
</tr>
<tr>
<td>MAMC (cm)</td>
<td>26.0 ± 3.4</td>
<td>23.2 ± 3.3</td>
<td>23.6 ± 3.1</td>
</tr>
<tr>
<td>Handgrip strength (kg)</td>
<td>32 ± 12</td>
<td>24 ± 9</td>
<td>23 ± 7</td>
</tr>
</tbody>
</table>

<sup>1</sup>CVD, cardiovascular disease; LBMI, lean BMI; FBMI, fat BMI; MAMC, midarm muscle circumference; RBCs, red blood cells; IGF-I, insulin-like growth factor I; hs-CRP, high-sensitivity C-reactive protein; IL-6, interleukin 6.

<sup>2</sup>Two-factor multivariate ANOVA (MANOVA). Significant (<i>P < 0.05</i>) effects are given for sex (S), appetite (A), and the sex × appetite (S × A) interaction.

<sup>3</sup>Median; range in parentheses (all such values).

<sup>4</sup>No MANOVA interaction was found.

<sup>5</sup>No difference was found by chi-square test: 1.4; <i>P = 0.95</i>.

<sup>6</sup>No difference was found by chi-square test: 2.1; <i>P = 0.84</i>.

<sup>7</sup>No difference was found by chi-square test: 5.5; <i>P = 0.85</i>.

<sup>8</sup>Mean ± SD (all such values).

A novel finding of this study is the sex differences in the relation between handgrip strength among patients with poor appetite. Indeed, handgrip strength was recently proposed not only as a marker of body lean muscle mass but also as a prognostic factor and a nutrition-monitoring tool in patients undergoing dialysis (26). Although sociological sex differences and different cultural backgrounds may be present and may explain why a larger proportion of women in the current study than in the study by Burrows et al (17) reported poor appetite, the finding that both men and women exhibit decreasing serum concentrations of albumin, creatinine, urea, and IGF-I across appetite categories indicates that both groups are indeed malnourished. This observation is corroborated by the finding that adjustment of the analysis for sex did not affect the predictive value of appetite on the survival of patients on hemodialysis. However, our results indicate that the loss of handgrip strength is more pronounced among men with poor appetite than among women and that the patient’s sex is a contributing factor to the degree of appetite reported among these patients.

Several studies have found sex differences in the regulation of appetite (16). Feeding behavior corresponding to the ovarian hormone cycle (27, 28) and a decrease in food intake are associated with elevated estradiol concentrations (27, 29). These sex differences apparently include both hormonal effects, which depend on gonadal function and estrogen concentrations, and lifelong effects, which arise directly from genetic differences or from the effects of gonadal hormones early in life (16). The existence of sex-specific orexigenic and anorexigenic mechanisms in response to inflammation was recently suggested in rats (30) and may imply differences in the up-regulation of leptin and ghrelin, which
The inflammation present in uremia stimulates leptin expression in anorexic patients (31). Furthermore, recent data have shown that tumor necrosis factor rate (8, 9). Indeed, anorexic patients undergoing peritoneal dialysis contribute to a weaker anorexic response (lower plasma leptin) and a weaker orexic response (poorer feeding and lower plasma ghrelin) in male rats. The cause of anorexia among patients with CKD is probably multifactorial. However, it is plausible that proinflammatory cytokines may play an important role by acting on the central nervous system to alter the release or function (or both) of several key neurotransmitters, thereby altering both appetite and metabolic rate (8, 9). Indeed, anorexic patients undergoing peritoneal dialysis have higher concentrations of tumor necrosis factor α than do non-anorexic patients (31). Furthermore, recent data have shown that the inflammation present in uremia stimulates leptin expression (32, 33) by signaling through the central melanocortin system (34), although it also affects ghrelin concentrations (35, 36). Because men on dialysis who have inflammation seem to have a worse survival than do women with the same condition (13), it is plausible to hypothesize that the differences in the inflammatory burden between men and women (37) may differently influence the feeding behavior.

Sex hormones may also contribute to the different severity of symptoms associated with poor appetite in men and women. In fact, inflammation-induced anorexia was reported to be more severe among male rats (38), and previous reports suggest that estradiol and progesterone have inhibitory effects under both basal (39, 40) and inflammatory (41) conditions, whereas progesterone injections decreased the severity of anorexia among female rats (38). It is interesting that nephrectomized male rats developed anemia and malnutrition, whereas matched females were free from these symptoms, which shows sex divergences in expression and alteration of several sex hormone receptors in the kidney tissue (42). Moreover, the use of low doses of progestagen megestrol acetate was shown to improve the nutritional and inflammatory status, as well as anorexia, in maintenance dialysis patients (43, 44). We did not assess estrogen concentrations in the current study; however, whereas most of the women investigated were postmenopausal, it may be that continuous estrogen protection during menstruating years and other genetic differences yielded a physiology that is better protected against the inflammatory burden of CKD (13).

The Women’s Health Initiative showed that the administration of estrogen and progesterin during 3 y in 400 postmenopausal women resulted in less loss of lean soft tissue mass and a lower ratio of trunk to leg fat mass than were seen in postmenopausal control subjects (45). This observation is of interest for CKD patients, because a gain in fat mass in this population is associated with improved survival (46). In agreement with this finding, markers of muscle mass are poor predictors of survival in CKD women but not in men (47). Because sex differences involve not only metabolic and neural pathways that are responsible for

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**FIGURE 2.** Mean (±SEM) concentrations of high-sensitivity C-reactive protein (hs-CRP; A), interleukin 6 (IL-6; B) in relation to fat body mass index (FBMI), and insulin-like growth factor I (IGF-I; C) in relation to BMI among self-reported appetite categories in 223 patients undergoing prevalent hemodialysis. Differences across appetite categories were assessed by ANOVA or by Kruskal-Wallis test for the nonparametric variables: P = 0.007 for hs-CRP; P = 0.04 for FBMI; P = 0.002 for IL-6; P < 0.0001 for IGF-I; and P = 0.005 for BMI. This analysis comprised 124 patients reporting good appetite, 65 patients reporting sometimes bad appetite, and 34 patients reporting poor appetite.

**FIGURE 3.** Mean (±SEM) concentrations of high-sensitivity C-reactive protein (hs-CRP) in relation to handgrip strength among self-reported appetite categories in male and female patients undergoing prevalent hemodialysis. Multivariate ANOVA showed a significant appetite × sex interaction for handgrip strength values. Differences in handgrip strength across appetite categories were assessed by ANOVA: P = 0.0006 for men and P = 0.6 for women. The men reported good (n = 80), sometimes bad (n = 35), and poor (n = 12) appetites, and the women reported good (n = 44), sometimes bad (n = 30), and poor (n = 22) appetites.
controlling the systemic inflammatory response but also differences in the immune systemic response, we hypothesize that sex hormone protection in women may contribute to a lower susceptibility to the inflammation-induced anorexia associated with CKD.

Some limitations of the present study should be considered. First, the results may have been influenced because the self-reported appetite intensity and anthropometric measurements were assessed on the day of hemodialysis. Second, patients with poor appetite had longer dialysis vintage time than did the other patients, which indicates that long-term dialysis itself may be another cause of loss of appetite. However, adjustment for dialysis vintage did not affect survival. Third, the relatively low number of patients may have masked other interactions in inflammatory and nutritional markers that would have strengthened our hypothesis, which should be assessed in bigger cohorts. Finally, that fact that this was a cross-sectional analysis may limit the value of the study. Thus, the present study does not provide a mechanistic explanation for the effect of sex differences on the regulation of the severity of symptoms during anorexia. Studies that provide such an explanation will add substantially to the strength of our findings.

In conclusion, our findings confirm the usefulness of self-reports of appetite level as a predictor of outcome for patients undergoing hemodialysis. Because sex may determine the severity of symptoms, such as handgrip strength, among patients who report a poor appetite, this study also supports the hypothesis that uremic men may be more susceptible than women to inflammation-induced anorexia. Altogether, we speculate that the mechanisms behind anorexia in women differ from those in men, perhaps involving the cardioprotective effects of sex hormones.

We thank our support staff members, Ann Dreiman-Lif (clinical study coordinator), Annika Nilsson and Anki Emmoth (clinical studies), Björn Anderstam (biochemical analysis coordinator), and Monica Ericsson and Ann-Christin Bragfors-Helin (biochemical analysis), for their efforts on behalf of the study. JJC, ARQ, JA, and PS were responsible for the study concept and design. SS-J, PB, and OH were involved in patient recruitment and data collection. CMA, MES, and SK contributed to the laboratory procedures. JJC, ARQ, CMA, and MES were involved in analysis and data interpretation. ARQ and JJC were responsible for the statistical analyses. JJC, ARQ, JA, and SK wrote the first draft of the manuscript, which was reviewed by all coauthors. BL and AA participated administratively, technically, and materially in the study and acted as study supervisors. BL is affiliated with Baxter Healthcare Inc. None of the other authors had any personal or financial conflict of interest.

REFERENCES


### TABLE 2

Significant predictors of self-reported appetite in a multinomial logistic regression including 223 prevalent hemodialysis patients

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>Lower</th>
<th>Upper</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept of poor appetite</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept of sometimes bad appetite</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-I (&gt; compared with &lt; 159 ng/mL)</td>
<td>3.59</td>
<td>2.05</td>
<td>6.27</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Inflammation (hs-CRP &lt; compared with &gt; 10 mg/L)</td>
<td>2.39</td>
<td>1.38</td>
<td>4.15</td>
<td>0.002</td>
</tr>
<tr>
<td>Sex (women compared with men)</td>
<td>0.41</td>
<td>0.24</td>
<td>0.72</td>
<td>0.002</td>
</tr>
<tr>
<td>Age (&lt; compared with 66 y)</td>
<td>0.85</td>
<td>0.49</td>
<td>1.46</td>
<td>0.55</td>
</tr>
</tbody>
</table>

The model included self-reported appetite as the dependent variable and all factors significantly associated with the dependent variable in univariate analysis. IGF-I, insulin-like growth factor I; hs-CRP, high-sensitivity C-reactive protein. Pseudo $r^2 = 0.19; P < 0.0001$.

FIGURE 4. Unadjusted (A) and adjusted (B) survival of patients in each of the 3 appetite categories. Adjustment for potential confounding factors [age, sex, inflammation [C-reactive protein (CRP) > 10 mg/L], dialysis vintage, and Davies comorbidity index] was conducted with the Cox proportional hazards model. This analysis comprised 124 patients reporting good appetite, 65 patients reporting sometimes bad appetite, and 34 patients reporting poor appetite followed for ≤30 mo.


Choline-related supplements improve abnormal plasma methionine-homocysteine metabolites and glutathione status in children with cystic fibrosis

Sheila M Innis, A George F Davidson, Stepan Melynk, and S Jill James

ABSTRACT

Background: Liver triacylglycerol accumulation and oxidative stress are common in cystic fibrosis (CF) and also occur in choline deficiency. Previously, we showed an association between elevated plasma homocysteine, reduced ratios of S-adenosylmethionine to S-adenosylhomocysteine (SAM:SAH) and of phosphatidylcholine to phosphatidylethanolamine, and phospholipid malabsorption in children with CF.

Objective: The objective was to address a possible relation between altered methionine-homocysteine metabolism and choline metabolism in children with CF.

Design: Children with CF were assigned without bias to supplementation with 2 g lecithin/d (n = 13), 2 g choline/d (n = 12), or 3 g betaine/d (n = 10) for 14 d. Plasma concentrations of methionine, adenosine, cysteine, cysteinyl-glycine, glutathione, glutathione disulfide (GSSG), and fatty acids; SAM:SAH; and red blood cell phospholipids were measured within each group of children with CF before and after supplementation. Plasma from healthy children without CF (n = 15) was analyzed to obtain reference data.

Results: Children with CF had higher plasma homocysteine, SAH, and adenosine and lower methionine, SAM:SAH, and glutathione:GSSG than did children without CF. Supplementation with lecithin, choline, or betaine resulted in a significant increase in plasma methionine, SAM, SAM:SAH, and glutathione:GSSG and a decrease in SAH (n = 35). Supplementation with choline or betaine was associated with a significant decrease in plasma SAH and an increase in SAM:SAH, methionine, and glutathione:GSSG. Supplementation with lecithin or choline also increased plasma methionine and SAM.

Conclusion: We showed that dietary supplementation with choline-related compounds improves the low SAM:SAH and glutathione redox balance in children with CF. 


KEY WORDS Choline, betaine, phospholipids, ratio of glutathione to glutathione disulfide, GSH:GSSG, cystic fibrosis

INTRODUCTION

The cystic fibrosis (CF) gene maps on chromosome 7 and encodes the CF transmembrane conductance regulator (CFTR), a protein that spans the plasma membrane surface of epithelial cells and some intracellular membranes (1–4). When activated by cyclic AMP and protein kinase, CFTR opens to form an ATP-gated channel to allow chloride to enter the cell (3, 4). The most common mutation, which occurs in ≥70% of patients with CF, is a 3–base pair deletion encoding a phenylalanine at position 508 (ΔF508) of the CFTR. Impaired exocrine pancreatic function with reduced secretion of pancreatic enzymes and sodium bicarbonate results in malabsorption of nutrients in 85–90% of patients with CF (5). In the remaining patients, enzyme secretion is present, although sodium bicarbonate and fluid secretion are impaired. Clinical management of patients with CF with pancreatic enzyme insufficiency involves pancreatic enzyme replacements, which greatly improves but does not completely correct the fat malabsorption (6–9). CF is also accompanied by several clinical complications, including hepatic steatosis for which neither the cause nor the connection to defective CFTR is clear (10–12).

CF-associated liver disease includes fatty infiltration of hepatocytes and focal biliary fibrosis or cirrhosis, which are believed to be multifactorial and to involve biochemical changes (11, 13). Oxidant damage and impaired glutathione metabolism were also extensively described in CF and may play a role in the pathophysiology of the disease (13–18). Hepatic triacylglycerol accumulation is a well-known feature of choline deficiency and is believed to be explained by failure of adequate phosphatidylcholine synthesis to support secretion of triacylglycerols from the liver in VLDL (19, 20). In addition, reduced glutathione, the most important intracellular antioxidant in animal cells (21), is reduced in the liver of choline-deficient animals (22). Choline deficiency also results in decreased betaine, which is an important source of methyl groups for remethylation of homocysteine (23). Phosphatidylcholine synthesis occurs through 2 pathways: the cytidine diphosphocholine pathway, in which preformed choline is converted to phosphatidylcholine by cytidine diphosphocholine, and by sequential transfer of methyl groups from methionine by S-adenosylmethionine (SAM) to phosphatidylethanolamine in the reaction catalyzed by

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3 Reprints not available. Address correspondence to SM Innis, Child and Family Research Institute, University of British Columbia, 950 West 28th Avenue, Vancouver, BC V5Z 4H4, Canada. E-mail: sinnis@cw.bc.ca. Received December 7, 2005. Accepted for publication October 27, 2006.
The product of PEMT is $5\text{-adenosylhomocysteine} (\text{SAH})$, which is converted to homocysteine by SAH hydroxylase. Homocysteine can be remethylated to methionine by methionine synthase in the pathway requiring 5-methyltetrahydrofolate (MTHF) or by betaine-homocysteine methyltransferase, with the use of methyl groups from betaine (25–27). Alternatively, homocysteine can enter the transsulfuration pathway that leads to cysteine, the precursor of glutathione (25, 27). Previously, we showed elevated plasma homocysteine, a low methionine concentration, a low ratio of SAM to SAH (SAM:SAH), and an inverse association between plasma homocysteine and phosphatidylcholine in children with CF (6, 28). More recently, we showed low plasma choline, as well as betaine and dimethylglycine in children with CF (29). In the present studies, we provide evidence of a functional relation between the choline-methyl pool and altered methionine-homocysteine metabolism and oxidant-antioxidant balance through the demonstration of an increase in plasma SAM:SAH and the ratio of glutathione to glutathione disulfide (glutathione: GSSG) after supplementation of children with CF with sources of choline.

SUBJECTS AND METHODS

Experimental design and subjects

This was a study of 3 separate supplements involving children with CF who were outpatients of the CF Clinic at the British Columbia Children’s Hospital (BCCH). The children were enrolled after description of the project to the child and his or her parents at a CF clinic appointment. The children were then assigned without bias to 1 of 3 choline-related supplements. Body weight and height were measured, and routine blood work, including liver enzymes, hematology, serum zinc, selenium, and vitamins A and E, was completed as part of the clinic appointment, with additional blood collected for this study (day 0). The children were asked to return to the hospital after taking the assigned supplement for 14 d, at which time a second blood sample was collected. Routine hematology and clinical chemistry were done as part of the clinic visit on blood samples collected on study day 0 by the Hematopathology and Clinical Chemistry laboratories at the BCCH. CF genotype, sex, birth date, and medications and supplements were recorded from chart data. Forty children and their parents signed the informed consent, and 35 children completed the 14-d supplementation and provided blood samples before (day 0) and after (day 14) supplementation. Five children withdrew within 5 d of commencing the supplements because they did not want to continue taking the supplements or to return for blood sampling. Blood samples were collected from healthy children without CF ($n$ = 15) to provide reference data for the laboratory measures; clinical chemistry and hematology tests were not done for the reference children. This study was approved by the University of British Columbia Clinical Screening Committee for Research and Other Studies Involving Human Subjects and the Children’s and Women’s Hospital Research Coordinating Committee. Approval for the use of betaine in this study was also obtained from Health Canada. All the parents and children provided written informed consent.

Supplements

The children with CF were assigned to receive phospholipid (lecithin, $2 \times 1 \text{g}$), choline ($2 \times 925 \text{mg}$), or betaine ($3 \times 1 \text{g}$) daily for 14 d. The lecithin used was soy lecithin providing 23% phosphatidylcholine and 20% phosphatidylethanolamine, the
TABLE 1
Baseline characteristics of children with cystic fibrosis who took supplements for 14 d1

<table>
<thead>
<tr>
<th></th>
<th>All children (n = 35)</th>
<th>Lecithin (n = 13)</th>
<th>Choline (n = 12)</th>
<th>Betaine (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mo)</td>
<td>128.4 ± 7.62</td>
<td>123.2 ± 11.9</td>
<td>124.9 ± 13.7</td>
<td>139.9 ± 13.6</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boy (n)</td>
<td>23</td>
<td>9</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Girl (n)</td>
<td>12</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Weight (z score)</td>
<td>−0.29 ± 0.14</td>
<td>−0.33 ± 0.28</td>
<td>−0.31 ± 0.21</td>
<td>−0.18 ± 0.30</td>
</tr>
<tr>
<td>Height (z score)</td>
<td>−0.30 ± 0.16</td>
<td>−0.16 ± 0.28</td>
<td>−0.39 ± 0.31</td>
<td>−0.34 ± 0.27</td>
</tr>
<tr>
<td>BMI (z score)</td>
<td>−0.31 ± 0.15</td>
<td>−0.32 ± 0.29</td>
<td>0.00 ± 0.20</td>
<td>−0.04 ± 0.33</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>130.0 ± 180.0</td>
<td>129.1 ± 1.9</td>
<td>130.7 ± 3.7</td>
<td>132.6 ± 3.6</td>
</tr>
<tr>
<td>Vitamin A (μmol/L)</td>
<td>1.37 ± 0.05</td>
<td>1.36 ± 0.09</td>
<td>1.27 ± 0.05</td>
<td>1.51 ± 0.14</td>
</tr>
<tr>
<td>Vitamin E (μmol/L)</td>
<td>22.2 ± 1.21</td>
<td>24.0 ± 1.80</td>
<td>22.4 ± 2.22</td>
<td>19.6 ± 2.58</td>
</tr>
<tr>
<td>Zinc (μmol/L)</td>
<td>12.3 ± 0.30</td>
<td>12.4 ± 0.57</td>
<td>12.2 ± 0.62</td>
<td>12.4 ± 0.35</td>
</tr>
<tr>
<td>Selenium (μmol/L)</td>
<td>1.64 ± 0.03</td>
<td>1.59 ± 0.06</td>
<td>1.66 ± 0.06</td>
<td>1.68 ± 0.06</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>214 ± 11</td>
<td>209 ± 11</td>
<td>193 ± 20</td>
<td>236 ± 21</td>
</tr>
<tr>
<td>Aspartate transaminase (U/L)</td>
<td>35.6 ± 2.48</td>
<td>32.3 ± 1.61</td>
<td>40.6 ± 6.16</td>
<td>33.2 ± 2.51</td>
</tr>
<tr>
<td>Alanine transaminase (U/L)</td>
<td>27.7 ± 1.86</td>
<td>24.4 ± 3.46</td>
<td>30.6 ± 3.40</td>
<td>28.3 ± 2.70</td>
</tr>
<tr>
<td>γ Glutamyl transaminase (U/L)</td>
<td>19.6 ± 0.77</td>
<td>20.4 ± 1.52</td>
<td>18.8 ± 0.92</td>
<td>19.6 ± 1.68</td>
</tr>
<tr>
<td>Lactate dehydrogenase (U/L)</td>
<td>604 ± 22.4</td>
<td>609 ± 34.4</td>
<td>628 ± 38.6</td>
<td>566 ± 41.7</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/L)</td>
<td>2.6 ± 0.27</td>
<td>2.7 ± 0.40</td>
<td>2.1 ± 0.51</td>
<td>3.0 ± 0.57</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>3.1 ± 0.11</td>
<td>3.2 ± 0.19</td>
<td>2.9 ± 0.19</td>
<td>3.1 ± 0.24</td>
</tr>
</tbody>
</table>

1 Blood samples were collected on day 0, before supplementation began. No significant differences between groups were observed by ANOVA.
2 ± SEM (all such values).

choline was citrus-flavored choline chloride providing 925 mg choline/5 mL (Life Extension, Fort Lauderdale, FL), and betaine was from Sigma Aldridge Chemical Co (product no. B2629; Oakville, Canada). All the supplements were packaged by the BCCH pharmacy in coded bottles. Every bottle had a 2-wk diary printed on the label. The children were asked to take the supplements with meals and to tick on the diary each time the supplement was taken. The lowest observed adverse effect level, based on mild hypotension and fishy body odor, for choline is 7.5 g/d (30), whereas betaine has no reported adverse effects in clinical settings. The lowest observed adverse effect level, based on mild hypotension and fishy body odor, for choline is 7.5 g/d (30), whereas betaine has no reported adverse effects in clinical settings.

Analytic methods

Plasma and red blood cell (RBC) lipids were extracted, then the polar and nonpolar lipids were separated by HPLC, quantified with the use of an evaporative light-scattering detector, and recovered with the use of a fraction collector (28, 32). The fatty acid components in the plasma phosphatidylcholine and RBC phosphatidylcholine, phosphatidylethanolamine, and phosphatidylerine were separated and quantified with the use of gas-liquid chromatography (33). Plasma thiols were measured with the use of reversed phase ion-pairing HPLC coupled to a coulometric electrochemical detector to allow simultaneous quantification, without derivatization of methionine, SAM, homocysteine, cystathionine, cysteine, cysteinylic-glycine, and glutathione as described previously (34, 35). Plasma and RBC folate and plasma vitamin B-12 were quantified by radioimmunoassay, and triacylglycerols and cholesterol were determined with the use of enzymatic methods (28).

Statistical analysis

All statistical analyses were performed with the use of SPSS for WINDOWS (version 10.0; SPSS, Chicago, IL). Data are presented as means ± SEMs. We used one-factor analysis of variance to compare the plasma thiols, glutathione, and GSSG concentrations, without derivatization of methionine, SAH, SAM, homocysteine, cystathionine, cysteine, cysteinylic-glycine, and glutathione as described previously (34, 35). Plasma and RBC folate and plasma vitamin B-12 were quantified by radioimmunoassay, and triacylglycerols and cholesterol were determined with the use of enzymatic methods (28).

RESULTS

The characteristics of the children with CF who completed the 14-d supplementation are shown in Table 1. Of the 35 children who completed the study, 26 were homozygous and 7 were heterozygous for the ΔF508 mutation; 1 child was homozygous for the G85E mutation, and 1 child was G542X/G551D.
significant difference was observed in age; z scores for height, weight, or body mass index; or the plasma measures of liver function, hemoglobin, vitamins A or E, zinc, selenium, triacylglycerols, or cholesterol between the 3 groups of children with CF who took the different supplements (Table 1). All of the children with CF had pancreatic insufficiency and were taking pancreatic enzyme replacements (500–2500 U lipase/kg per meal). None of the children were taking folic or vitamin B-6 antagonists, had chronic renal disease or malignancy, or were taking N-acetyl cysteine or any medications reported to increase homocysteine (36). Amino thiols, such as penicillamine, can reduce plasma homocysteine; however, no significant differences were observed in any of the plasma metabolites measured in this study between the children taking no antibiotics (n = 22) and children taking antibiotics (n = 13). No medication changes were made in any child during participation in this study.

The plasma concentrations of metabolites for methionine cycle and transsulfuration pathway in the children with CF before supplementation and in the reference children are shown in Table 2. The children with CF had higher plasma homocysteine, SAH, adenosine, and GSSG but lower methionine, glutathione, SAM:SAH, and glutathione:GSSG than did the reference children.

The plasma metabolite concentrations in the children with CF before and after 14 d of supplementation with lecithin, choline, or betaine are shown in Tables 3, 4, and 5, respectively. Supplementation with lecithin was associated with a significant increase in plasma methionine and SAM, but it had no statistically significant effect on the concentration of any other metabolite. Children with CF assigned to choline had significantly higher plasma concentrations of methionine and SAM, higher SAM:SAH and glutathione:GSSG, and lower plasma concentration of SAH after supplementation than before supplementation (Table 4). In the group of children with CF assigned to betaine, the plasma concentration of methionine was higher, SAM:SAH and glutathione:GSSG were lower, and the concentrations of homocysteine, SAH, adenosine, and cysteine were higher on day 14 than on day 0 of the study (Table 5).

The analysis of the RBC phospholipids showed no significant differences in the concentration of individual phospholipids for phosphatidylcholine, sphingomyelin, lysophosphatidylcholine, or phosphatidylglycerol between the children with CF and the group of reference children. However, RBC phosphatidylethanolamine was higher (10.26 ± 3.4 and 8.63 ± 2.7 mg/L) and the ratio of phosphatidylcholine to phosphatidylethanolamine was lower (0.63 ± 0.2 and 0.84 ± 0.02; P < 0.05) in the children with CF (n = 35) than in a group of reference children (n = 15).

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Plasma thiols in children with cystic fibrosis (CF) and in a group of reference children without CF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reference children (n = 15)</td>
</tr>
<tr>
<td>Methionine (μmol/L)</td>
<td>25.9 ± 1.43</td>
</tr>
<tr>
<td>Hcy (μmol/L)</td>
<td>6.06 ± 0.35</td>
</tr>
<tr>
<td>SAM (nmol/L)</td>
<td>91.0 ± 6.23</td>
</tr>
<tr>
<td>SAH (nmol/L)</td>
<td>15.8 ± 1.16</td>
</tr>
<tr>
<td>SAM:SAH</td>
<td>6.22 ± 0.06</td>
</tr>
<tr>
<td>Adenosine (μmol/L)</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Cysteine (μmol/L)</td>
<td>209 ± 9.25</td>
</tr>
<tr>
<td>Cyst-glyc (μmol/L)</td>
<td>45.0 ± 1.77</td>
</tr>
<tr>
<td>Free GSH (μmol/L)</td>
<td>2.07 ± 0.17</td>
</tr>
<tr>
<td>Free GSSG (μmol/L)</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>GSH:GSSG</td>
<td>16.7 ± 2.78</td>
</tr>
</tbody>
</table>

1 Hcy, homocysteine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Cyst-glyc, cysteinyl-glycine; GSH, glutathione; GSSG, glutathione disulfide. Blood samples for children with CF were collected on day 0 of the study before commencing any supplementation.

2 Difference between the children with CF and the reference group of children.

3 x ± SEM (all such values).

4 Significantly different from reference children, P < 0.05 (one-factor ANOVA).

<table>
<thead>
<tr>
<th>TABLE 3</th>
<th>Effect of supplementation with lecithin for 14 d on plasma thiols in children with cystic fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lecithin (n = 13)</td>
</tr>
<tr>
<td>Day 0</td>
<td>Day 14</td>
</tr>
<tr>
<td>Methionine (μmol/L)</td>
<td>19.1 ± 1.23</td>
</tr>
<tr>
<td>Hcy (μmol/L)</td>
<td>8.56 ± 0.79</td>
</tr>
<tr>
<td>SAM (nmol/L)</td>
<td>83.1 ± 4.77</td>
</tr>
<tr>
<td>SAH (nmol/L)</td>
<td>25.6 ± 2.96</td>
</tr>
<tr>
<td>SAM:SAH</td>
<td>3.93 ± 0.55</td>
</tr>
<tr>
<td>Adenosine (μmol/L)</td>
<td>0.42 ± 0.07</td>
</tr>
<tr>
<td>Cysteine (μmol/L)</td>
<td>244 ± 9.60</td>
</tr>
<tr>
<td>Cyst-glyc (μmol/L)</td>
<td>43.0 ± 2.6</td>
</tr>
<tr>
<td>Free GSH (μmol/L)</td>
<td>1.62 ± 0.21</td>
</tr>
<tr>
<td>Free GSSG (μmol/L)</td>
<td>0.46 ± 0.09</td>
</tr>
<tr>
<td>GSH:GSSG</td>
<td>4.91 ± 0.81</td>
</tr>
</tbody>
</table>

1 All values are x ± SEM. Hcy, homocysteine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Cyst-glyc, cysteinyl-glycine; GSH, glutathione; GSSG, glutathione disulfide. The effect of supplementation was analyzed by using 2-factor ANOVA.

2 Significantly different from day 0, P < 0.05 (paired t test).

<table>
<thead>
<tr>
<th>TABLE 4</th>
<th>Effect of supplementation with choline for 14 d on plasma thiols in children with cystic fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Choline (n = 12)</td>
</tr>
<tr>
<td>Day 0</td>
<td>Day 14</td>
</tr>
<tr>
<td>Methionine (μmol/L)</td>
<td>21.6 ± 1.03</td>
</tr>
<tr>
<td>Hcy (μmol/L)</td>
<td>7.83 ± 0.32</td>
</tr>
<tr>
<td>SAM (nmol/L)</td>
<td>81.3 ± 2.27</td>
</tr>
<tr>
<td>SAH (nmol/L)</td>
<td>29.4 ± 2.63</td>
</tr>
<tr>
<td>SAM:SAH</td>
<td>2.97 ± 0.23</td>
</tr>
<tr>
<td>Adenosine (μmol/L)</td>
<td>0.52 ± 0.08</td>
</tr>
<tr>
<td>Cysteine (μmol/L)</td>
<td>238 ± 9.8</td>
</tr>
<tr>
<td>Cyst-glyc (μmol/L)</td>
<td>39.2 ± 2.00</td>
</tr>
<tr>
<td>Free GSH (μmol/L)</td>
<td>1.53 ± 0.17</td>
</tr>
<tr>
<td>Free GSSG (μmol/L)</td>
<td>0.38 ± 0.06</td>
</tr>
<tr>
<td>GSH:GSSG</td>
<td>5.02 ± 0.74</td>
</tr>
</tbody>
</table>

1 All values are x ± SEM. Hcy, homocysteine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Cyst-glyc, cysteinyl-glycine; GSH, glutathione; GSSG, glutathione disulfide. The effect of supplementation was analyzed by using 2-factor ANOVA.

2 Significantly different from day 0, P < 0.05 (paired t test).
We found no statistically significant differences in the concentrations of individual phospholipids in the RBC membranes of the children with CF after 14 d of supplementation with lecithin, choline, or betaine (data not shown). However, in the group of children given choline, the RBC membrane ratio of phosphatidylcholine to phosphatidylethanolamine was lower (P < 0.05) on day 0 (0.52 ± 0.02) than on day 14 (0.91 ± 0.04) of supplementation, which was explained by a higher phosphatidylcholine and lower phosphatidylethanolamine concentration on day 14 (824 ± 3 and 1043 ± 70 mg/mL, respectively) than on day 0 (752 ± 23 and 929 ± 58 mg/L, respectively) of the study. The plasma phospholipid fatty acid analyses showed that, compared with the group of reference children (n = 15), the children with CF (n = 35) had significantly lower concentrations of linoleic acid (22.1 ± 0.50 and 25.4 ± 0.80 g/100 g fatty acids) and docosahexaenoic acid (2.31 ± 0.11 and 2.88 ± 0.30 g/100 g fatty acids) and significantly higher concentrations of eicosatrienoic acid (4.5 ± 0.15 and 3.74 ± 0.12 g/100 g fatty acids) and eicosapentaenoic acid (0.89 ± 0.5 and 0.70 ± 0.07 g/100 g fatty acids) (P < 0.05); arachidonic acid concentrations were not significantly different between the children with CF and the reference group (9.65 ± 0.29 and 9.79 ± 0.49 g/100 g fatty acids, respectively). We found no significant differences in n-6 (linoleic acid, eicosatrienoic acid, arachidonic acid) or n-3 (docosahexaenoic acid, eicosapentaenoic acid) fatty acid concentrations between day 14 and day 0 in the group of children with CF who took lecithin, choline, or betaine (data not shown).

**DISCUSSION**

This study was the first to show that supplementation with choline-related compounds alters plasma methionine-homocysteine cycle metabolites, which leads to increased plasma methionine and SAM:SAH and to an improved glutathione antioxidant status in children with CF. This study was based on our recent work that showed increased plasma homocysteine and decreased SAM:SAH in children with CF, which was not explained by inadequate folate or vitamin B-12 status but was associated with a reduced plasma ratio of phosphatidylcholine to phosphatidylethanolamine (6, 28). The metabolism of choline is interrelated with the methionine-homocysteine cycle at 2 steps: 1) through the methylation of phosphatidylethanolamine to form phosphatidylcholine with the use of methyl groups from methionine by SAM with the generation of SAH and 2) through the betaine-dependent remethylation of homocysteine to methionine (Figure 1). Although methylation of phosphatidylethanolamine is an important source of phosphatidylcholine and plasma homocysteine (37–39), an increase in SAH results in inhibition of PEMT and a decrease in plasma phosphatidylcholine and choline (40, 41). In animals, choline deficiency results in a decrease in hepatic betaine synthesis and SAM, which suggests that the folate-dependant remethylation of homocysteine may not fulfill the requirements for regeneration of methionine when the betaine-dependent remethylation of homocysteine is limited by choline deficiency (42). Other studies have shown that plasma betaine concentrations are inversely associated with plasma homocysteine and increase after methionine loading (43, 44), which suggests that choline-derived betaine is important in methionine-homocysteine metabolism in humans. Our recent study showed low plasma choline and betaine in children with CF (29).

The results of the present study show that supplementation with choline, betaine, or lecithin increased the low plasma methionine and that supplementation with choline or betaine decreased the elevated SAH and increased the plasma SAM:SAH in children with CF. These results are consistent with the interdependence of the methionine-homocysteine cycle with choline metabolism (26) and the low choline status of children with CF (29). Children with CF who took choline or betaine had significantly higher plasma SAH and SAM:SAH after 14 d of supplementation. However, the plasma SAM concentrations were significantly higher after 14 d of supplementation when compared with day 0 for those children with CF who took either choline or lecithin but not in those children with CF who took betaine. Dietary phospholipid requires digestion by pancreatic phospholipase A2 before absorption as lysophospholipid, which is followed by reacylation in the enterocyte or direct transport to the liver bound to albumin. Previously, we showed an increased fecal excretion of choline phosphoglycerides in children with CF, regardless of supplementation with pancreatic enzymes (26, 45). In addition, the phospholipid supplement used in our studies provided ≈0.3 g choline compared with 1.85 g choline in the choline supplement. These differences may explain in part why our results show a statistically significantly lower plasma SAH concentration and a higher SAM:SAH after 14 d of supplementation with choline but not with lecithin in children with CF.

Previously, we reported an elevated concentration of homocysteine and decreased SAM:SAH in children with CF (6, 28), which raises the possibility that inhibition of PEMT could contribute to reduced de novo choline synthesis and a subsequent low choline status, exacerbated by chronic phosphatidylcholine malabsorption and increased phosphatidylcholine turnover (6, 28, 29, 45). Plasma homocysteine concentrations depend on the rate of homocysteine formation from methionine and the rate of removal by remethylation to methionine by either methionine synthase, which requires MTHF or betaine-homocysteine methyltransferase, or the rate of entry to the transulfuration pathway, which is regulated by cystathionine β-synthase (25–27). SAM

In Nizam and SEK, the plasma concentrations of hexadecanoate, octadecenoate, and palmityl-oleate were significantly increased, whereas the plasma concentrations of stearoyl-stearate, dihomo-γ-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid were significantly decreased in patients with SEK compared with controls. These results suggest that the plasma concentrations of specific fatty acids are differentially regulated in patients with SEK.
serves to regulate the metabolism of homocysteine through allo-
sthetic inhibition of MTHF reductase and activation of cystathione β-synthase (25). In the presence of low SAM, as shown in children with CF, the remethylation of homocysteine is fa-
vored, and decreased cystathione β-synthase activity serves to con-
serve methionine. The increase in plasma methionine and SAM
in children given lecithin or choline may reflect a sparing of methionine (and SAM) for phosphatidylcholine synthesis by
the PEMT pathway, which possibly also explains the lack of a
statistically significant increase in plasma SAM after supple-
mentation with betaine (Figure 1). Studies in animals, however,
have shown that supplementation with betaine increases the re-
cycling of homocysteine to methionine (46), and, in clinical
practice, betaine is efficacious in reducing elevated homocys-
teine (47). The latter studies are consistent with the findings of
the present study that show both a lower plasma concentration of
homocysteine and a higher plasma concentration of methionine
in children with CF in the betaine group on day 14 than on day 0
(Table 3), which suggests that supplementation with betaine was
efficacious in supporting an increase in the betaine-dependent
regeneration of homocysteine to methionine. However, we de-
tected no significant difference in the plasma homocysteine con-
centration between day 14 and day 0 in children with CF assigned
to take choline or lecithin. Possibly, the latter results are ex-
plained by greater efficacy of preformed betaine than of its chlo-
line precursor in supporting the remethylation of homocysteine.

Recent studies have led to an increased understanding of the
importance of oxidative stress and glutathione system dysfunc-
tion in many diseases (21), including many of the complications
of CF (13–18), particularly those associated with the immune
system (13–18, 48, 49). Glutathione plays a pivotal role in de-
toxifying reactive molecules generated by mitochondria and mi-
crosomal CYP-450 enzymes and in maintaining reduced sul-
dryl groups on molecules involved in cell proliferation, apopto-
sis, energy production, and calcium homeostasis (50). Glutathione is also exported to the extracellular spaces, such as
the epithelial lining fluid of the lung, and to immune cells where
it serves an important antioxidant function (21, 22). The intra-
cellular glutathione status depends on precursor availability, the
rate of glutathione oxidation to GSSG, and the capacity to recycle
GSSG back to glutathione at the expense of NADPH (21, 22).
In our study, we measured the plasma glutathione, most of which is
derived by export from the liver; GSSG, however, is exported
from cells as an important mechanism to maintain a high intra-
cellular glutathione:GSSG (36). The glutathione:GSSG is often
used as an indicator of the cellular redox status and is >10 under
most normal physiologic conditions (21). In our study, the gluat-
athione:GSSG in children with CF was <5 and ≈25% that of the
control children, consistent with numerous reports of oxidative
stress in CF (13–18). We found that glutathione:GSSG was
≈45–60% higher after 14 d of supplementation than before
supplementation with choline-related metabolites, which was
statistically significant in those children with CF given choline or
betaine. Because SAM activates cystathione β-synthase activ-
ity (21) and because our results showed that supplementation
with methyl groups from choline increased SAM and SAM:
SAH, it is possible that entry of homocysteine to the transsulfu-
ration pathway and glutathione synthesis was also increased.
Supplementation with betaine of children with CF, however, was
associated with a significant decrease in the elevated plasma
concentrations of adenosine and homocysteine, both of which
when elevated are associated with increased oxidative stress.
Whether a reduction in oxidative stress secondary to reduced
adenosine and homocysteine is related to the increased gluta-
thane:GSSG in the children in our study given supplements of
betaine is unclear, as is the reason for the accompanying decrease
in the plasma cysteine, which is the metabolic precursor to glu-
thaione (21).

The results of this study provide evidence that supplemen-
tation with choline-related metabolites (choline, lecithin, and be-
taine) may improve the abnormal membrane lipid composition
in children with CF. Supplementation with choline, possibly by
providing increased choline to support phosphatidylcholine syn-
thesis, was associated with a significant increase in the RBC
membrane ratio of phosphatidylcholine to phosphatidylethano-
lamine. However, neither the physiologic significance nor the
relevance to other cell membranes can be addressed by our re-
results.

In summary, our studies provide evidence that the metabolism
of methionine, homocysteine, and choline are interrelated in
humans. We have shown that choline or betaine supplementation
of children with mutations in CFTR, resulting in the clinical
spectrum of CF, results in an increased plasma concentration of
methionine and increased SAM:SAH and glutathione:GSSG.
Possibly, chronic malabsorption of choline-containing phospho-
lipids in CF results in depletion of choline, which may be further
compromised by a decrease in de novo glutathione synthesis result-
ning from a low SAM:SAH in children with CF (6, 28, 29, 45)
(Table 2). Supplementation with methyl groups as choline may
provide an effective intervention through conservation of methi-
onine and increased SAM, which could have beneficial clinical
effects related to choline availability and oxidative stress in pa-
tients with CF. Alternatively, supplementation with betaine
could provide an effective intervention to reduce homocysteine
and oxidative stress and to increase the recycling of homocys-
teine to methionine. Further studies are needed to consider the
efficacy of different supplements, such as choline and betaine,
and to identify the appropriate doses in studies of sufficient
duration to consider the clinical relevance of these interventions.

We thank the parents and children who participated in this study and the
staff at British Columbia Children’s Hospital for facilitating this study. RA
Milner is acknowledged for her expert assistance in designing the study and
conducting the statistical analysis.

SMI was the principal investigator and helped with grant funding, with
the study concept and design, and with the manuscript and data preparation.
AGFD participated as the clinician scientist in patient selection, patient
enrollment, and collection of clinical information. SJJ and SM measured the
plasma thiols. All of the authors contributed to the review and revision of
the manuscript. None of the authors had a conflict of interest.

REFERENCES
1. Bradbury NA. Intracellular CFTR. Localization and function. Physiol
fibrosis gene: chromosome walking and jumping. Science 1989;245:
3. Reisin IL, Prat AG, Abraham EH, et al. The cystic fibrosis transmem-
brane conductance regulator is a dual ATP and chloride channel. J Biol
rectifying chloride channels through an autocrine mechanism involving
Continuous intake of polyphenolic compounds containing cocoa powder reduces LDL oxidative susceptibility and has beneficial effects on plasma HDL-cholesterol concentrations in humans\textsuperscript{1,2}

Seigo Baba, Naomi Osakabe, Yoji Kato, Midori Natsume, Akiko Yasuda, Toshimi Kido, Kumiko Fukuda, Yuko Muto, and Kazuo Kondo

ABSTRACT

Background: Cocoa powder is rich in polyphenols such as catechins and proanthocyanidins and has been shown in various models to inhibit LDL oxidation and atherogenesis.

Objective: We examined whether long-term intake of cocoa powder alters plasma lipid profiles in normocholesterolemic and mildly hypercholesterolemic human subjects.

Design: Twenty-five subjects were randomly assigned to ingest either 12 g sugar/d (control group) or 26 g cocoa powder and 12 g sugar/d (cocoa group) for 12 wk. Blood samples were collected before the study and 12 wk after intake of the test drinks. Plasma lipids, LDL oxidative susceptibility, and urinary oxidative stress markers were measured.

Results: At 12 wk, we measured a 9% prolongation from baseline levels in the lag time of LDL oxidation in the cocoa group. This prolongation in the cocoa group was significantly greater than the reduction measured in the control group (\textminus 13%). A significantly greater increase in plasma HDL cholesterol (24%) was observed in the cocoa group than in the control group (5%). A negative correlation was observed between plasma concentrations of HDL cholesterol and oxidized LDL. At 12 wk, there was a 24% reduction in dityrosine from baseline concentrations in the cocoa group. This reduction in the cocoa group was significantly greater than the reduction in the control group (\textminus 1%).

Conclusion: It is possible that increases in HDL-cholesterol concentrations may contribute to the suppression of LDL oxidation and that polyphenolic substances derived from cocoa powder may contribute to an elevation in HDL cholesterol.

KEY WORDS Cocoa, LDL oxidative susceptibility, HDL cholesterol, catechins

INTRODUCTION

Evidence indicates that oxidation of LDL has a pathogenic role in the development of atherosclerosis (1). Uptake of oxidized LDL by macrophages and smooth muscle cells leads to the formation of fatty streaks, a key event in early atherosclerosis. These vascular lesions accumulate large amounts of lipids, such as cholesterol ester. In addition, oxidized LDL induces the expression of adhesion molecules in monocytes, such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, and also increases production of growth factors by smooth muscle cells and fibroblasts (2, 3). These findings suggest that inhibition of LDL oxidation may prevent atherosclerotic lesions.

Prospective studies, such as the Framingham Heart Study, Multiple Risk Factor Intervention Trial, Coronary Primary Prevention Trial, Lipid Research Clinics Prevalence Mortality Follow-up Study, and the Prospective Cardiovascular Münster study all reported a negative correlation between plasma HDL cholesterol and cardiovascular disease (4, 5). It has been proposed that HDL may inhibit LDL oxidation by various mechanisms (6). There is also clinical evidence of this suppressive effect of HDL: a study conducted in 270 patients with coronary heart disease showed a negative correlation between plasma concentrations of HDL cholesterol and oxidized LDL (7). These results suggest that one of the protective mechanisms of high HDL concentrations is to cause inhibition of LDL oxidation. Prospective studies have shown a negative correlation between the consumption of plant polyphenols and mortality from both coronary and ischemic heart diseases (8, 9), with studies conducted in both rats and humans reporting that intake of these polyphenols suppressed oxidation of LDL (10).

Cacao beans are used as an ingredient in cocoa and chocolate and are known to be rich in polyphenols, such as catechin, epicatechin, procyanidin B2 (dimer), procyanidin C1 (trimer), cinnamaldehyde A2 (tetramer), and other oligomeric procyanidins (11). A Dutch study revealed chocolate is a major source of catechins, especially in the younger population (12). In previous studies, we showed intake of polyphenolic-rich fractions derived from cocoa powder increased the resistance of LDL to oxidation and suppressed the formation of atherosclerosis in hypercholesterolemic rabbits (13). Studies we carried out in healthy human subjects also showed intake of dairy cocoa powder enhanced the resistance of LDL to oxidation (14, 15). To further delineate the role of cocoa powder in atherogenesis protection, we examined...
the effects of cocoa intake on plasma concentrations of oxidized LDL and lipids and the urinary oxidative stress markers 8-oxo-7,8-dihydro-2′-deoxyguanosine and lipid hydroperoxide-derived protein modification in normocholesterolemic and mildly hypercholesterolemic human subjects.

SUBJECTS AND METHODS

Materials

Cocoa powder was prepared by roasting, cracking, and compressing fermented and dried cacao beans imported from Ecuador. The general composition of the cocoa powder was as follows (units/100 g): 23.0 g protein, 11.5 g fat, 23.1 g carbohydrate, 26.9 g fiber, 7.7 g minerals, 377 mg epicatechin, 135 mg catechin, 158 mg procyanidin B2, 96.1 mg procyanidin C1, 2192 mg theobromine, and 470 mg caffeine. The catechin, epicatechin, procyanidin B2, and procyanidin C1 content in the powder was analyzed by an HPLC method (11). Other reagents used in the study were commercially available products of analytic and HPLC grade.

Subjects

Twenty-five healthy Japanese male subjects participated in the study. The study was approved by and performed under the guidelines of the ethics committee of Tomisaka Hospital, and informed consent was obtained from each of the subjects before commencement of the study. All subjects were of normal body weight and were nonsmokers with no evidence of chronic disease. None of the subjects consumed >25 mL alcohol/d or were taking other medications, antioxidants, or vitamin supplements. The study group had a mean (±SEM) age of 38 ± 1 y, a mean body weight of 64 ± 1 kg, and a mean body mass index (BMI) of 22.1 ± 0.2 kg/m². The concentration ranges of plasma total, LDL, and HDL cholesterol in the subjects were 4.65–6.41 mmol/L, 2.46–4.92 mmol/L, and 0.75–2.60 mmol/L, respectively.

Experimental design

The subjects were divided into 2 groups according to BMI, and plasma total, LDL, and HDL cholesterol concentrations and were then instructed to consume one of the following test drinks daily for 12 wk: 12 g sugar/d (control group) or a mixture of 26 g cocoa powder and 12 g sugar/d (cocoa group). The cocoa powder was consumed as a beverage after the addition of hot water, with the test drinks being consumed twice each day: before noon and during the afternoon. At baseline and at 12 wk, the subjects fasted for 12 h, and then blood samples were collected from the intermediate cubital vein into a tube containing EDTA-2Na. At the same times during the study, 24-h urine samples were collected from 0900 the day before the blood collection until 0900 of the day of the collection. Body weight, blood pressure, and heart rate were also measured at the beginning and end of the study. Home deliveries of food were made to each subject to ensure that the same foods were consumed in the 3 d before collection of the blood and urine samples. In addition, to maintain their normal diets, the subjects kept complete dietary records throughout the study. The 3-d food records were analyzed with the Excel Food-Frequency Questionnaire (Kenpakusha, Tokyo, Japan) on days 1–3, 26–28, 54–56, and 80–82 of each dietary period. The subjects were also requested to avoid all other cacao products and to lead their usual lifestyle throughout the study.

Plasma LDL oxidative susceptibility

Plasma LDL oxidative susceptibility was measured as the lag time of conjugated diene production formed by a radical generator. The lag time was determined by using methods described previously (16, 17). LDL was isolated from plasma by single-spin density gradient centrifugation (417 000 g, 40 min, 4 °C) by using a ultracentrifuge (Optima TLX; Beckman Instruments, Inc, Palo Alto, CA). The density gradient was adjusted to 1 mL plasma by the addition of 0.325 g potassium bromide. The protein concentration of the LDL fraction was measured by using the Micro BCA protein assay reagent kit (Pierce, Rockford, IL), followed immediately by the assay of LDL oxidation. Isolated LDL samples were diluted with phosphate-buffered saline (PBS; pH 7.4) to a concentration of 100 µg LDL protein/mL, followed by incubation for 250 min at 37 °C with the radical generator 2,2′-azobisis(4-methoxy-2,4-dimethylvaleronitrile) (200 µmol/L). The formation of conjugated dienes was monitored continuously every 3 min by the change in absorbance at 234 nm with the use of a spectrophotometer (DU800; Beckman Instruments Inc, Palo Alto, CA). The lag time in this reaction, expressed in minutes, provided an assessment of LDL oxidation and was calculated by determining the point of intersection of the baseline and propagation phase of the absorbance curve.

Plasma lipids and oxidative LDL

Plasma VLDL-, LDL-, and HDL-cholesterol concentrations at baseline and at 12 wk were measured by a rapid electrophoresis scanning automated system (Helena Laboratories, Saitama, Japan) with the use of agarose-gel electrophoresis (18). Triacylglycerol was assayed by a standard laboratory technique (BML Inc, Tokyo, Japan). The monoclonal antibody mAb 4E6 was used to quantify the concentration of oxidized LDL in plasma at baseline and at 12 wk (19). This assay was carried out by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Mercodia Oxidized LDL ELIZA; Mercodia AB, Uppsala, Sweden), according to the manufacturer’s instructions.

Urinary oxidative stress markers

The following variables were measured as markers of urinary oxidative stress at baseline and at 12 wk: 8-oxo-7,8-dihydro-2′-deoxyguanosine, N°-(hexanoyl)lysine, dityrosine, bromotyrosine, and dibromotyrosine. Urine 8-oxo-7,8-dihydro-2′-deoxyguanosine concentrations were measured by using a commercially available ELISA kit (8OHDG check, JαlCA; Nikken SEIL Co, Shizuoka, Japan) according to the manufacturer’s instructions, whereas quantification of N°-(hexanoyl)lysine in urine was carried out by using liquid chromatography–tandem mass spectrometry (LC–MS) as described previously (20). Quantification of oxidized modified forms of tyrosine was also carried out by using liquid chromatography–tandem mass spectrometry LC–MS (Y Kato, N Dozaki, T Nakamura, et al, unpublished observations, 2004).

Urinary catechin and epicatechin

The amounts of catechin and epicatechin in the urine samples collected at baseline and at 12 wk were analyzed by LC-MS according to methods described previously (21, 22). These earlier reports showed that ingested catechin and epicatechin are present in plasma and urine primarily as various metabolites, such as glucuronide conjugated forms, sulfate conjugated forms,
or both. In our study, we therefore measured catechin and epicatechin metabolites in urine after hydrolysis treatment with glucuronidase and sulfatase (Sulfatase type H-5; Sigma, St Louis, MO) (23). The sum of each catechin or epicatechin metabolites was calculated to determine the total amounts of catechin and epicatechin excreted in the urine.

Safety measurements

The following variables were measured in the blood samples collected at baseline and at 12 wk: plasma total protein, albumin, glucose, uric acid, urea nitrogen, creatinine, free fatty acids, phospholipids, total bilirubin, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltranspeptidase, alkaline phosphatase, lactate dehydrogenase, sodium, potassium, chloride, and calcium. Urine samples collected at baseline and at 12 wk were used for qualitative analysis of proteinuria, glucosuria, urobilinogen, and occult blood. All these variables were assayed by using standard laboratory techniques (BML Inc, Tokyo, Japan).

Statistics

The data were expressed as means ± SEMs. The change from baseline (12 wk – baseline) in the control and cocoa groups were compared by using repeated-measures analysis of variance and unpaired t tests to assess whether a significant group × time interaction had occurred. A mixed model analysis was used to examine the interaction between 2 risk factors with time and the risk factors acting as the independent variables. If a significant interaction was found, separate correlations were calculated at baseline and 12 wk using Pearson’s correlation analysis. A lag time. The changes were significantly different between the 2 groups (P < 0.001).

RESULTS

Subject characteristics and dietary records

Mean BMI, systolic blood pressure, diastolic blood pressure, and heart rate at baseline in the control and cocoa groups were 22.1 ± 0.3 and 22.1 ± 0.4 kg/m², 117 ± 2 and 124 ± 3 mm Hg, 79 ± 2 and 77 ± 2 mm Hg, and 71 ± 2 and 77 ± 2 beats/min, respectively. Mean BMI, systolic blood pressure, diastolic blood pressure and heart rate at 12 wk in the control and cocoa groups were 21.5 ± 0.3 and 21.6 ± 0.4 kg/m², 120 ± 3 and 122 ± 2 mm Hg, 77 ± 2 and 75 ± 2 mm Hg, and 72 ± 3 and 77 ± 3 beats/min, respectively. No significant differences were observed in any of these variables between the 2 groups (BMI, P = 0.730; systolic blood pressure, P = 0.221; diastolic blood pressure, P = 0.934; and heart rate, P = 0.961). The baseline values of plasma biochemical variables, lipids, oxidized LDL concentrations, LDL susceptibility, and urinary oxidative stress markers did not differ significantly between the 2 groups. No subject reported any adverse events resulting from cocoa intake at the interviews conducted throughout the study. No significant differences in daily mean energy and nutrient intake were observed between the 2 groups during the 3-d periods that dietary records were collected (Table 1).

Plasma LDL oxidative susceptibility

Changes in the susceptibility of LDL to oxidation expressed as lag time are shown in Table 2. In the control group, we observed a 19.8% reduction in lag time at 12 wk compared with baseline. In contrast, in the cocoa group we found a 9.4% prolongation in lag time. The changes were significantly different between the 2 groups (P < 0.001).

Plasma lipids and oxidative LDL

The profiles of plasma total, VLDL, LDL, and HDL cholesterol and of triacylglycerol at baseline and at 12 wk are summarized in Table 3. Plasma total, VLDL-, LDL-, and HDL-cholesterol concentrations at baseline were not significantly different in the 2 groups. In the cocoa group, there was a 23.4% increase in HDL cholesterol at 12 wk compared with baseline concentrations. This increase in the cocoa group was significantly greater (P < 0.001) than that measured in the control group (5.1%). The concentrations of LDL cholesterol at 12 wk were reduced by 12.6% and 4.5% in the cocoa and control groups, respectively. However, there was no significant difference in the magnitude of these reductions between the 2 groups. Also, no significant difference in total and VLDL cholesterol and triacylglycerol concentrations was observed between the 2 groups.

The plasma concentrations of oxidized LDL at baseline and at 12 wk are shown in Table 2. No significant difference in oxidized LDL concentrations was observed between the 2 groups.

Urinary oxidative stress markers

The concentrations of 8-oxo-7,8-dihydro-2′-deoxyguanosine, Nα-(hexanoyl)lysine, dityrosine, bromotyrosine, and dibromotyrosine in urine at baseline and at 12 wk are shown in Table 4. At 12 wk, there was a 23.6% reduction in dityrosine from baseline concentrations in the cocoa group. This reduction in the cocoa group was significantly greater (P < 0.05) than the reduction in the control group (−1.1%). A nonsignificant trend of decreasing Nα-(hexanoyl)lysine concentrations was observed in the cocoa group compared with the control group (group × time interaction, P = 0.06).

Urinary catechin and epicatechin

The 24-h urinary excretion of catechin and epicatechin at baseline and at 12 wk are shown in Figure 1. After 12 wk, we
Baseline values did not differ significantly between the 2 groups. The cocoa group than in the control group (catechin and epicatechin being significantly higher at 12 wk in group. These increases resulted in the urinary excretion of both observed an approximate 8-fold increase in catechin excretion in the cocoa group than in the control group ($P < 0.001$ for both).

### Safety measurements

The concentrations of plasma biochemical variables at baseline and at 12 wk are summarized in Table 5. No significant differences were observed between the 2 groups for any of these variables. Other variables, including plasma total protein, albumin, urea nitrogen, free fatty acids, phospholipid, sodium, potassium, chloride, calcium, and all other urinary variables listed in the Methods section were within the normal range in all the subjects during the study (data not shown).

#### Correlation between plasma and urinary factors

The relation between plasma oxidized LDL (in U/L plasma) and LDL and HDL cholesterol at baseline and at 12 wk is shown in Figure 2. A negative correlation was observed between plasma oxidized LDL and HDL cholesterol at 12 wk ($r = -0.460$, $P = 0.021$), whereas the correlation between plasma oxidized LDL and LDL cholesterol was not significant. The correlations between plasma oxidized LDL (in U/L plasma) and HDL cholesterol and urinary concentrations of catechin and epicatechin are shown in Figure 3. A negative correlation was

### TABLE 2

Plasma oxidized LDL and LDL oxidative susceptibility in the 2 groups at baseline and at 12 wk

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 12</th>
<th>Value</th>
<th>$P^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma oxidized LDL (U/L plasma)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>73.7 ± 8.8$^3$</td>
<td>70.9 ± 4.1</td>
<td>-2.8 ± 6.6</td>
<td>NS</td>
</tr>
<tr>
<td>Cocoa group</td>
<td>70.8 ± 5.1</td>
<td>57.7 ± 3.2</td>
<td>-13.2 ± 4.3</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Plasma oxidized LDL (U · mmol LDL cholesterol$^{-1}$ L plasma$^{-1}$)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>20.9 ± 2.5</td>
<td>22.2 ± 2.1</td>
<td>1.27 ± 1.67</td>
<td>NS</td>
</tr>
<tr>
<td>Cocoa group</td>
<td>20.7 ± 1.6</td>
<td>19.8 ± 1.5</td>
<td>0.95 ± 1.12</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Lag time (min)$^4$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>58.6 ± 3.8</td>
<td>47.0 ± 2.3</td>
<td>-11.6 ± 3.8</td>
<td>NS</td>
</tr>
<tr>
<td>Cocoa group</td>
<td>57.4 ± 3.1</td>
<td>62.8 ± 1.8</td>
<td>5.4 ± 2.9$^5$</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

$^1$ $n = 12$ and 13 for the control and cocoa groups, respectively. The control group received 0 g cocoa; the cocoa group received 26 g cocoa powder/d. Baseline values did not differ significantly between the 2 groups.

$^2$ Unpaired $t$ test. Statistical analyses with the use of repeated-measures ANOVA provided essentially the same results.

$^3$ $x$ ± SEM (all such values).

$^4$ LDL oxidative susceptibility was measured by the lag time of conjugated diene production formed with the radical generator 2,2’-azobis (4-methoxy-2,4-dimethylvaleronitrile).

$^5$ Significantly different from the control group, $P < 0.05$.

### TABLE 3

Plasma total cholesterol, VLDL-cholesterol, LDL-cholesterol, HDL-cholesterol, and triacylglycerol concentrations in the 2 groups at baseline and 12 wk

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 12</th>
<th>Value</th>
<th>$P^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cholesterol (mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>5.27 ± 0.14$^2$</td>
<td>5.17 ± 0.19</td>
<td>-0.09 ± 0.15</td>
<td>NS</td>
</tr>
<tr>
<td>Cocoa group</td>
<td>5.28 ± 0.16</td>
<td>5.09 ± 0.17</td>
<td>-0.19 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td><strong>VLDL cholesterol (mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>0.39 ± 0.05</td>
<td>0.38 ± 0.05</td>
<td>-0.01 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Cocoa group</td>
<td>0.41 ± 0.06</td>
<td>0.35 ± 0.04</td>
<td>-0.06 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td><strong>LDL cholesterol (mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>3.52 ± 0.19</td>
<td>3.36 ± 0.23</td>
<td>-0.16 ± 0.14</td>
<td>NS</td>
</tr>
<tr>
<td>Cocoa group</td>
<td>3.50 ± 0.16</td>
<td>3.06 ± 0.22</td>
<td>-0.43 ± 0.14</td>
<td>NS</td>
</tr>
<tr>
<td><strong>HDL cholesterol (mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>1.36 ± 0.15</td>
<td>1.43 ± 0.15</td>
<td>0.08 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Cocoa group</td>
<td>1.37 ± 0.11</td>
<td>1.69 ± 0.13</td>
<td>0.31 ± 0.05$^4$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Triacylglycerol (mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>0.95 ± 0.10</td>
<td>1.09 ± 0.13</td>
<td>0.14 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td>Cocoa group</td>
<td>1.05 ± 0.13</td>
<td>1.06 ± 0.12</td>
<td>0.01 ± 0.13</td>
<td>NS</td>
</tr>
</tbody>
</table>

$^1$ $n = 12$ and 13 for the control and cocoa groups, respectively. The control group received 0 g cocoa; the cocoa group received 26 g cocoa powder/d. Baseline values did not differ significantly between the 2 groups.

$^2$ Unpaired $t$ test. Statistical analyses with the use of repeated-measures ANOVA provided essentially the same results.

$^3$ $x$ ± SEM (all such values).

$^4$ Significantly different from the control group, $P < 0.05$. 

$^5$ Significantly different from the control group, $P < 0.05$. 

The concentrations of plasma biochemical variables at baseline and at 12 wk are summarized in Table 5.
observed between plasma oxidized LDL and urinary epicatechin at 12 wk \( (r = -0.433, P = 0.030). \) A trend toward a nonsignificant, negative correlation was also observed between plasma oxidized LDL and urinary catechin at 12 wk \( (r = -0.375, P = 0.064). \)

FIGURE 1. Mean (±SEM) urinary excretion of catechin and epicatechin in the control (■, \( n = 12 \)) and cocoa (■, \( n = 13 \)) groups at baseline and 12 wk. The baseline values did not differ significantly between the 2 groups. At 12 wk, there was a significant increase in catechin and epicatechin concentrations in the cocoa group compared with the control group \( (P < 0.001). \) *Significantly different from the control group, \( P < 0.001 \) (unpaired \( t \) test).

### DISCUSSION

The present study indicated that consumption of cocoa powder containing polyphenolic substances at a dosage of 26 g/d for 12 wk increased the resistance of LDL to oxidation and also raised HDL-cholesterol concentrations in plasma in normocholesterolemic and mildly hypercholesterolemic humans.

It has been reported that there is a positive correlation between the resistance of LDL to oxidation and the severity of coronary atherosclerosis in humans and that susceptibility of LDL to oxidation is significantly higher in affected familial combined hyperlipidemic subjects than in unaffected subjects \( (24, 25). \) Our study showed that intake of cocoa powder had a favorable effect on the susceptibility of LDL to oxidation. This is consistent with other investigations that showed a positive correlation between inhibition of LDL oxidation and the amount of total phenolic compounds derived from wine or the concentration of major polyphenols in cocoa powder such as catechin, epicatechin, and their oligomers \( (26, 27). \) These results suggest that polyphenols from cocoa powder may contribute to the resistance of LDL to oxidation. In the present study, catechins were detected in urine in the group that consumed cocoa, although catechins in plasma were not measured. Studies conducted in both rat and humans have shown that after oral administration of cocoa powder, catechin, epicatechin, and procyanidin dimers \( (B2 and B5) \) are absorbed and appear in the plasma \( (21, 28–31). \) Similarly, the polyphenol concentration in LDL particles has been shown to be elevated 2 wk after intake of red wine and that the decrease in LDL susceptibility correlated positively with polyphenol concentration \( (32). \) In addition, Hayek et al \( (33) \) showed that both catechin and quercetin were present in LDL after intake of red wine and that these flavonoids bound to the LDL particle by formation of glycosidic bonds. Another study suggested that flavonoids may associate with apolipoprotein B because of their capacity to bind to proteins \( (34). \) These results suggest

<table>
<thead>
<tr>
<th>8-OHdG (µmol/24 h)</th>
<th>Baseline</th>
<th>Week 12</th>
<th>Value</th>
<th>( P^{2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>32.7 ± 7.0(^d)</td>
<td>32.6 ± 4.8</td>
<td>−0.1 ± 6.6</td>
<td>NS</td>
</tr>
<tr>
<td>Cocoa group</td>
<td>38.6 ± 6.1</td>
<td>38.1 ± 6.8</td>
<td>−0.5 ± 8.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>( N-N' ) (Hexanoyl)lysine (µmol/24 h)</th>
<th>Baseline</th>
<th>Week 12</th>
<th>Value</th>
<th>( P^{2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>34.0 ± 4.2</td>
<td>28.6 ± 3.7</td>
<td>−5.4 ± 2.3</td>
<td>NS</td>
</tr>
<tr>
<td>Cocoa group</td>
<td>42.3 ± 4.5</td>
<td>26.0 ± 3.3</td>
<td>−16.3 ± 4.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dityrosine (µmol/24 h)</th>
<th>Baseline</th>
<th>Week 12</th>
<th>Value</th>
<th>( P^{2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>74.1 ± 6.2</td>
<td>73.3 ± 5.4</td>
<td>−0.8 ± 5.2</td>
<td>NS</td>
</tr>
<tr>
<td>Cocoa group</td>
<td>91.7 ± 8.1</td>
<td>70.1 ± 7.6</td>
<td>−21.6 ± 8.5(^*)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bromotyrosine (µmol/24 h)</th>
<th>Baseline</th>
<th>Week 12</th>
<th>Value</th>
<th>( P^{2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>34.5 ± 5.5</td>
<td>34.8 ± 5.1</td>
<td>0.4 ± 3.2</td>
<td>NS</td>
</tr>
<tr>
<td>Cocoa group</td>
<td>50.1 ± 10.0</td>
<td>31.7 ± 3.8</td>
<td>−18.4 ± 9.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dibromotyrosine (µmol/24 h)</th>
<th>Baseline</th>
<th>Week 12</th>
<th>Value</th>
<th>( P^{2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>25.0 ± 3.4</td>
<td>32.7 ± 4.2</td>
<td>7.7 ± 3.2</td>
<td>NS</td>
</tr>
<tr>
<td>Cocoa group</td>
<td>38.9 ± 4.9</td>
<td>45.8 ± 6.5</td>
<td>6.8 ± 6.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

\( ^{1} n = 12 \) and 13 for the control and cocoa groups, respectively. The control group received 0 g cocoa; the cocoa group received 26 g cocoa powder/d.

\( ^{2} \) Unpaired \( t \) test. Statistical analyses with the use of repeated-measures ANOVA provided essentially the same results.

\( ^{3} \bar{x} ± SEM \) (all such values).

\( ^{d} \) Significantly different from the control group, \( P < 0.05. \)
that some of the polyphenols absorbed from cocoa powder may be incorporated onto the surface of LDL particles and that these polyphenols increase the resistance of LDL to oxidation by either scavenging chain-initiating oxygen radicals or chelating transitional metal ions (35). Polyphenols located on the surface of LDL particles may also have a sparing and recycling effect on fat-soluble antioxidants, such as \( \alpha \)-tocopherol, by supplying hydrogen to fat-soluble antioxidants, which in turn provide hydrogen to lipid peroxide radicals (36).

Marsu et al (37) reported that HDL-cholesterol concentrations increased by 11% and 14% after 3-wk intake of dark chocolate or dark chocolate enriched with cocoa polyphenols, respectively.

![FIGURE 2](image_url) Plasma LDL and HDL cholesterol versus plasma oxidized LDL in the control (baseline ○; 12 wk ●, \( n = 12 \)) and cocoa (baseline □; 12 wk ■, \( n = 13 \)) groups. The baseline values did not differ significantly between the 2 groups. Because there was a significant interaction between time and both plasma LDL and HDL cholesterol in the mixed model analysis, Pearson’s correlation coefficients were calculated for the baseline and 12-wk data. The slopes, correlation coefficients, and \( P \) values are shown in each of the figures.
The daily consumption of catechin monomers and procyanidins in their study was 270 mg with the dark chocolate and 420 mg with the polyphenol-enriched dark chocolate. These results indicated that the increase in plasma HDL-cholesterol concentrations caused by polyphenols was dose-related. Our study also showed that cocoa powder enhanced plasma HDL-cholesterol concentrations and that there was a nonsignificant trend toward a positive correlation between the excretion of urinary catechin and plasma HDL cholesterol. There is some evidence on in vivo absorption and metabolism of polyphenols in cocoa powder (28, 29, 38, 39). These findings suggest that absorbed catechins in cocoa powder may affect plasma HDL-cholesterol concentrations. Intake of flavonoids other than catechins, such as isoflavones, flavones (naringenin and hesperetin), and polyphenols in red wine, have also been shown to increase plasma HDL concentrations in both human and animal studies (40–43). Taken together, these results indicate that ingestion of polyphenols from sources other than cocoa powder may also affect plasma HDL-cholesterol concentrations. However, results from other studies on polyphenol supplementation support our finding that polyphenols in cocoa powder are responsible, in part, for the increase we observed in plasma HDL-cholesterol concentrations.

It has been reported that increased HDL leads to suppression of LDL oxidation by promoting 1) inhibition of monocyte chemotaxis via monocyte chemotactic protein-1, 2) hydrolysis of lipid peroxide via paraoxonase, 3) reverse cholesterol transport via lecithin-cholesterol acyltransferase, and 4) direct inhibition of vascular endothelial activation via apolipoprotein A1 (44–47). Our study showed a negative correlation between plasma oxidized LDL and HDL cholesterol, whereas only a weak degree of correlation was observed between plasma oxidized LDL and LDL cholesterol. Holvoet et al (7) showed that plasma concentrations of oxidized LDL correlated inversely with HDL-cholesterol concentrations, whereas there was no relation between plasma concentrations of oxidized LDL and LDL cholesterol. Alternatively, catechins in cocoa powder have proven in vitro antioxidative activity, although it has been shown that catechins absorbed from cocoa powder are present mainly in the plasma as metabolites, such as conjugated forms, methylated forms, or both that may have decreased antioxidative activity (21, 48). These results suggest that catechins in cocoa powder may inhibit LDL oxidation not only by antioxidative mechanisms but also by other mechanisms. It is therefore possible that increased HDL-cholesterol concentrations caused by polyphenolic substances derived from cocoa powder may contribute to suppression of LDL oxidation.

The mechanisms by which polyphenolic compounds elevate plasma HDL-cholesterol concentrations remains unclear. One hypothesis is that apolipoprotein A1, the major protein component of HDL, has a role in increasing HDL cholesterol. Evidence supporting this possibility is that genistein was shown to increase the expression and production of apolipoprotein A1 in a human hepatoma cell line Hep G2 (49). Lamon-Fava et al (50) also showed that regulation of apolipoprotein A1 expression by genistein was mediated by the mitogen-activated protein kinase signaling pathway.

During lipid peroxidation, several aldehydes, such as 4-hydroxy-2-nonenal and malondialdehyde, are produced after degradation of lipid hydroperoxide. These reactive compounds...
are predisposed to react with proteins and aminolipids. Oxidized modified forms of lysine and tyrosine have been detected in human atherosclerotic plaque (51–53). Urinary excretion of \( N^0 \)-hexanoyllysine has also been shown to be significantly higher in patients with diabetes than in control subjects (20). In our study, intake of cocoa powder reduced the excretion of urinary dityrosine significantly and was also associated with a trend of lower \( N^0 \)-hexanoyllysine excretion compared with control subjects (\( P = 0.061 \)). The oxidative products measured in the present study are stable in urine and could therefore be useful markers for the diagnosis of oxidative stress in the body.

It has been reported that survival rate and the incidence of clinical disease and carcinogenicity remain unchanged in rats fed a diet containing 5% cocoa powder for 104 wk. Cocoa powder has also been shown to have no teratogenic or embryotoxic activity in rabbits (54, 55) and tested negative in short-term assays for genotoxicity (56). In addition, we also reported that intake of 26 g of cocoa powder for 12 wk in humans was not associated with abnormalities in blood and urine variables (15). In the present study, daily consumption of cocoa powder also had no significant influence on blood and urine variables, blood pressure, or BMI, and no adverse effects were reported to the doctors at the patient interviews. These results confirm the findings of previous studies regarding the safety of cocoa products.

Cocoa powder contains fiber and methylxanthines compounds such as caffeine and theobromine. However, in the present study, the control group drink did not control for the fiber, caffeine, and theobromine contents of the cocoa group drink. Wan et al (57) reported that cocoa powder and chocolate had favorable effects on LDL oxidative susceptibility and HDL-cholesterol concentrations compared with a control diet with similar fat, protein, carbohydrate, cholesterol, fiber, caffeine, and theobromine content. This result indicates that polyphenolic compounds from cocoa powder and chocolate may contribute to these favorable effects.

In conclusion, the present study showed that daily intake of cocoa powder decreased the susceptibility of LDL to oxidation and increased HDL-cholesterol concentrations in plasma in humans. Plasma HDL-cholesterol concentrations correlated negatively with plasma oxidized LDL, whereas plasma oxidized LDL concentrations correlated negatively with excretion of urinary epicatechin. It is possible that increases in HDL-cholesterol concentrations may contribute to suppression of LDL oxidation. Because polyphenolic substances derived from cocoa powder contribute to the elevation of HDL cholesterol, it would be anticipated that intake of polyphenol-rich foods, such as cocoa, tea, wine, fruit, and vegetables, should lead to a decrease in the incidence of arteriosclerotic disease. Moreover, it is irrefutable that a balanced daily diet is important for the promotion of human health.

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REFERENCES


Coffee intake and incidence of hypertension

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ABSTRACT

Background: The long-term longitudinal evidence for a relation between coffee intake and hypertension is relatively scarce.

Objective: The objective was to assess whether coffee intake is associated with the incidence of hypertension.

Design: This study was conducted on a cohort of 2985 men and 3383 women who had a baseline visit and follow-up visits after 6 and 11 y. Baseline coffee intake was ascertained with questionnaires and categorized into 0, >0–3, >3–6, and >6 cups/d. Hypertension was defined as a mean systolic blood pressure (SBP) ≥140 mm Hg over both follow-up measurements, a mean diastolic blood pressure (DBP) ≥90 mm Hg over both follow-up measurements, or the use of antihypertensive medication at any follow-up measurement.

Results: Coffee abstainers at baseline had a lower risk of hypertension than did those with a coffee intake of >0–3 cups/d (odds ratio [OR]: 0.54; 95% CI: 0.31, 0.92). Women who drank >6 cups/d had a lower risk than did women who drank >0–3 cups/d (OR: 0.67; 95% CI: 0.46, 0.98). Subjects aged ≥39 y at baseline had 0.35 mm Hg (95% CI: −0.59, −0.11 mm Hg) lower SBP per cup intake/d and 0.11 mm Hg lower DBP (95% CI: −0.26, 0.03 mm Hg) than did those aged <39 y at baseline, although the difference in DBP was not statistically significant.

Conclusions: Coffee abstinence is associated with a lower hypertension risk than is low coffee consumption. An inverse U-shaped relation between coffee intake and risk of hypertension was observed in the women. Am J Clin Nutr 2007;85:718–23.

KEY WORDS Coffee, hypertension, cohort study

INTRODUCTION

Coffee consumption has long been a suspected cause of hypertension, but the available evidence from various study designs is inconsistent. Many randomized experiments have been performed but with different coffee or caffeine intakes. In a recent meta-analysis of 16 trials with both coffee and caffeine interventions, we showed that for coffee trials with a median intake of 725 mL coffee/d there was a rise of 1.2 mm Hg in systolic blood pressure and of 0.5 mm Hg in diastolic blood pressure (1). These trials were designed for a short follow-up duration.

Most evidence on the relation between coffee and blood pressure stems from cross-sectional studies. This evidence, however, is inconsistent. Some of these studies showed a positive relation (2), no relation (3), or even an inverse relation (4). Such cross-sectional studies have important limitations with respect to causal inference.

Conclusive information about coffee as a cause for hypertension cannot be expected to come from randomized trials, because those would require unrealistically long-term interventions. Rather, long-term observational cohort studies will have to provide such information. There have been few follow-up studies on the relation between coffee intake and blood pressure or risk of hypertension (5, 6, 7). In 1017 young men, a small positive association between coffee intake and blood pressure rise over many years of follow-up was indicated to play a small role in the development of hypertension (6). In women participating in the Nurse’s Health Studies, an inverse U-shaped relation was recently found between hypertension and caffeine consumption, but no association was found with caffeinated coffee consumption (7).

Because the long-term longitudinal evidence for a relation between coffee intake and hypertension is relatively scarce, we used a Dutch cohort study to address that issue. This cohort allowed for studying the relation of baseline coffee intake to the incidence of persistent hypertension on the basis of repeatedly measured blood pressure levels in subjects at 5 y intervals during a follow-up of 11 y. Our specific research question was whether coffee intake in subjects who are not hypertensive is associated with the incidence of hypertension.

SUBJECTS AND METHODS

The design of the Doetinchem Cohort Study is described in detail elsewhere (8). Briefly, the subjects were inhabitants of the Dutch city of Doetinchem who had participated in 2 subsequent general population screening projects for chronic disease risk

1 From the Julius Center for Health Sciences and Primary Care, University Medical Center, Utrecht, Netherlands (CSPMU, PHMP, and DEG); the Center for Prevention and Health Services Research (WMMV), for Nutrition and Health (HBbDM, MO, and EJMF), and for Information Technology and Methodology (HCB), National Institute of Public Health and the Environment, Bilthoven, Netherlands; and the Division of Human Nutrition, Wageningen University, Wageningen, Netherlands (JMG).

2 The Doetinchem Cohort Study was financially supported by the Ministry of Public Health, Welfare, and Sports of the Netherlands and the National Institute of Public Health and the Environment, Bilthoven, Netherlands; and the Division of Human Nutrition, Wageningen University, Wageningen, Netherlands (JMG).

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factors: the Monitoring Project on Cardiovascular Disease Risk Factors [Peilstationsproject Hart- en Vaatziekten (PPHV)] (9) conducted between 1987 and 1991, and the Monitoring Project on Risk Factors for Chronic Diseases–European Prospective Investigation into Cancer and Nutrition (MORGEN-EPIC) (10) conducted between 1993 and 1997. The subjects were invited for a third separate visit between 1998 and 2002. The response rate was 62% at baseline, 78% at the first follow-up, and again 78% at the third follow-up. Respondents who attended the baseline and at least one of the follow-up examinations were included in the present analysis (n = 6368). The median follow-up time was 11 y. The study was approved by the Medical Ethics Committee of the Organization for Applied Scientific Research-Zeist, Netherlands. All subjects signed an informed consent form.

Dietary variables and exposure categories

Coffee intake at baseline of PPHV was estimated by the question “How many cups of coffee do you drink per day?,” a question on the type of coffee used (regular, decaffeinated, or other), and a question about the use of additives (none, milk, sugar, etc). In MORGEN-EPIC, the subjects were asked to indicate how frequently they usually drank coffee, the type of coffee (regular, instant, decaffeinated, or other), use of additives (sugar, milk, and type of milk) with color photographs to indicate the strength of coffee and the standard size of a cup equaling 125 g. In a food-frequency questionnaire, the respondents were instructed to record what, on average, they had eaten and drunk in the past year. The MORGEN-EPIC food-frequency questionnaire was also used in the third follow-up (11). Similar questions were asked about tea intake. Coffee intake at baseline was divided into 4 categories: 0 cups/d, >0–3 cups/d, >3–6 cups/d, and >6 cups/d. The category of >0–3 cups/d was chosen as the reference category rather than the non-coffee drinking category because it contained larger numbers of subjects and yielded more stable estimates.

At baseline of PPHV, the subjects filled out a mailed questionnaire about demography, family history of cardiovascular disease, other chronic disease (eg, diabetes mellitus), current medication use, prescribed diets, selected dietary habits, and reproductive history for women. Pregnant women were excluded from the study. Questionnaires were used to assess alcohol intake (glasses/d), smoking status (none, ever, or current smoking of cigarettes), educational level (low, medium, or high) based on highest educational level achieved, and occupational status (paid work, housekeeping, unemployed, or retired or other).

Anthropometric and biological variables

Body height was measured to the nearest 0.5 cm without shoes. Body weight was measured without shoes and heavy clothing to the nearest 0.1 kg.

At all visits, nonfasting blood samples were obtained by using a standardized protocol. Plasma total and HDL cholesterol were measured at the Clinical Chemistry Laboratory of the University Hospital “Dijkzigt” in Rotterdam, which is the Lipid Reference Laboratory for standardized cholesterol determinations in the Netherlands. Total cholesterol was measured enzymatically by using a Boehringer test kit (12). HDL-cholesterol concentrations were measured after precipitation of apolipoprotein B–containing lipoproteins with magnesium phosphotungstate (13).

Outcome measurements and definitions

In PPHV, blood pressure was measured by trained technicians using a random zero sphygmomanometer while the subject was in a sitting position. The cuff size (12 × 23 cm) was applied to the left upper arm. A larger cuff (15 × 33 cm) was used in 1.1% and a smaller cuff (9 × 18 cm) in 0.4% of all examined subjects. Systolic blood pressure was recorded at the appearance of sounds (first-phase Korotkoff) and diastolic blood pressure at the disappearance of sounds (fifth-phase Korotkoff). After the first measurement, the heart rate was measured for 30 s followed by a second blood pressure measurement. In MORGEN-EPIC and at the third visit, the blood pressure measurement procedure was identical to that performed in PPHV. No restrictions were made with regard to coffee drinking before the measurements were taken.

Hypertension was defined by using cutoffs according to the recommendations in the 7th report of the Joint National Committee (JNC) of the National Heart, Lung, and Blood Institute (14), which classifies stage 1 hypertension as a systolic blood pressure of 140–159 mm Hg or a diastolic blood pressure of 90–99 mm Hg and stage 2 hypertension as having systolic blood pressure of ≥160 mm Hg or diastolic blood pressure ≥100 mm Hg, use of antihypertensive medication, or both. To have sufficient numbers of hypertensives in each category of coffee intake, these categories of hypertension were pooled to at least JNC stage 1 hypertension. Persistent hypertension was defined as having a mean systolic blood pressure ≥140 mm Hg or a mean diastolic blood pressure ≥90 mm Hg calculated over both follow-up measurements at a 5-y interval, the use of antihypertensive medication at any of the follow-up measurements, or both. The association between baseline coffee intake and incident hypertension as defined above was assessed among those who did not have hypertension at baseline. No hypertension at baseline was defined as having a systolic blood pressure <140 mm Hg and a diastolic blood pressure <90 mm Hg and no use antihypertensive medication.

Statistical analysis

The association between baseline coffee intake and incident hypertension as defined above was assessed among those who did not have hypertension at baseline as defined above. Logistic regression was used with presence of persistent hypertension (yes or no) as the dependent variable and baseline coffee intake and confounders as independent variables. Furthermore, effects of changes in coffee intake as a predictor of change of blood pressure were examined. A repeated-measures analysis with time-varying covariates was used with changes between repeated blood pressure measurements as dependent variables and time-varying changes in coffee intake and confounders as independent variables. In all analyses, we adjusted for the following possible confounders: age, sex, body height and weight, smoking, alcohol intake, tea intake, educational level, occupational status, and total energy intake.

All analyses were expressed as measures of association with corresponding 95% CIs, regarding intervals not including the respective null values as statistically significant. Analyses were performed by using SPSS version 11.0 or SAS Proc Mixed for repeated-measures analysis (SPSS Inc, Chicago, IL).
Baseline characteristics of the study cohort (n = 6368)

<table>
<thead>
<tr>
<th></th>
<th>Men (n = 2985)</th>
<th>Women (n = 3383)</th>
<th>P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>40.7 ± 10.0</td>
<td>40.1 ± 10.3</td>
<td>&lt;0.0009</td>
</tr>
<tr>
<td>Body height (cm)</td>
<td>178.9 ± 6.8</td>
<td>165.9 ± 6.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>81.1 ± 10.6</td>
<td>67.8 ± 10.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>125.6 ± 13.6</td>
<td>117.5 ± 14.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>79.4 ± 10.3</td>
<td>75.5 ± 10.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.6 ± 1.1</td>
<td>5.4 ± 1.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.1 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Normotensive (%)</td>
<td>77.0</td>
<td>85.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Previous myocardial infarction (%)</td>
<td>0.9</td>
<td>0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>0.7</td>
<td>0.7</td>
<td>0.96</td>
</tr>
<tr>
<td>Current cigarette smoker (%)</td>
<td>34.9</td>
<td>33.8</td>
<td>0.35</td>
</tr>
<tr>
<td>Education level (%)</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Low</td>
<td>55.3</td>
<td>68.7</td>
<td></td>
</tr>
<tr>
<td>Middle</td>
<td>24.5</td>
<td>18.7</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>20.2</td>
<td>12.6</td>
<td></td>
</tr>
<tr>
<td>Occupational status (%)</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Paid work</td>
<td>86</td>
<td>43.1</td>
<td></td>
</tr>
<tr>
<td>Housekeeping</td>
<td>0.7</td>
<td>48.8</td>
<td></td>
</tr>
<tr>
<td>Unemployed or retired</td>
<td>8.6</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>4.7</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Coffee intake</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>0 cups/d4</td>
<td>2.9</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>≤3 cups/d</td>
<td>15.7</td>
<td>27.0</td>
<td></td>
</tr>
<tr>
<td>3–6 cups/d</td>
<td>53.1</td>
<td>53.9</td>
<td></td>
</tr>
<tr>
<td>&gt;6 cups/d</td>
<td>28.3</td>
<td>13.5</td>
<td></td>
</tr>
<tr>
<td>Coffee intake (g/d)</td>
<td>625 (125–3000)</td>
<td>500 (62.5–2500)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Decaffeinated coffee user (no.)</td>
<td>327</td>
<td>509</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Decaffeinated coffee intake (g/d)</td>
<td>375 (125–3125)</td>
<td>375 (62.5–1875)</td>
<td>0.03</td>
</tr>
<tr>
<td>Tea intake (g/d)</td>
<td>300 (150–3000)</td>
<td>300 (150–2250)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Alcohol intake (glasses/d)</td>
<td>1.1 (0–14)</td>
<td>0 (0–7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total energy intake (kJ)</td>
<td>8119.9 (1984.6)</td>
<td>6331.4 (1941.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Follow-up (y)</td>
<td>11.0 ± 0.18</td>
<td>11.0 ± 0.17</td>
<td></td>
</tr>
</tbody>
</table>

1 Differences in continuous data were tested by using Student’s t test; difference in median no. of glasses alcohol/d was tested by using Mann-Whitney U test; and differences in proportional data were tested by using chi-square tests.
2 ± SD (all such values).
3 Systolic blood pressure <140 mm Hg and diastolic blood pressure <90 mm Hg and no antihypertensive treatment (14).
4 1 cup = 125g.
5 Median; min-max in parentheses (all such values).

RESULTS

The baseline characteristics at the first round (1987 to 1991) of the Doetinchem cohort are shown in Table 1. The prevalence of hypertension, as defined in Methods, at baseline was 23% in the men and 14.5% in the women.

The associations between baseline coffee intake and the subsequent development of persistent hypertension among normotensives at baseline are shown in Table 2. The interaction between sex and coffee intake in relation to hypertension was borderline statistically significant (P = 0.08), and therefore we decided to do both sex-specific analyses and analysis of the total group. The unadjusted odds ratios indicated a lower risk among noncoffee drinkers than in those who drank >0–3 cups/d. After adjustment, this association was slightly attenuated but was still detectable in the total group. Furthermore, women who drank >6 cups/d had a lower risk of hypertension than did women who drank >0–3 cups/d. Among coffee drinking women, there was a statistically significant trend over coffee intake categories (P = 0.023). Because age played a central role in confounding adjustments, we further explored to what extent the association between baseline coffee intake and later blood pressure differed with age. In linear regression models with the mean systolic or diastolic blood pressure from the last 2 visits as the dependent variable and coffee intake (cups), age, and a coffee intake × age interaction term as independent variables, there was a statistically significant interaction for systolic blood pressure (P = 0.0001) as well as for diastolic blood pressure (P < 0.0001). The prevalence of hypertension, as defined in Methods, at baseline was 23% in the men and 14.5% in the women.
Table 2

Relative risk for persistent hypertension occurrence in 11 y follow-up by baseline categories of coffee intake in 5189 normotensive subjects in the study cohort

<table>
<thead>
<tr>
<th>Coffee intake</th>
<th>Total cohort</th>
<th>No hypertension</th>
<th>Hypertension</th>
<th>OR (95% CI)</th>
<th>OR adjusted (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n</td>
<td>n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 cups/d</td>
<td>65</td>
<td>59</td>
<td>6</td>
<td>0.40 (0.17, 0.96)</td>
<td>0.60 (0.24, 1.49)</td>
</tr>
<tr>
<td>&gt;0–3 cups/d</td>
<td>379</td>
<td>302</td>
<td>77</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>&gt;3–6 cups/d</td>
<td>1195</td>
<td>928</td>
<td>267</td>
<td>1.13 (0.85, 1.50)</td>
<td>1.08 (0.79, 1.47)</td>
</tr>
<tr>
<td>&gt;6 cups/d</td>
<td>658</td>
<td>515</td>
<td>143</td>
<td>1.09 (0.80, 1.49)</td>
<td>1.03 (0.72, 1.46)</td>
</tr>
<tr>
<td>Women</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 cups/d</td>
<td>166</td>
<td>155</td>
<td>11</td>
<td>0.38 (0.20, 0.71)</td>
<td>0.51 (0.26, 1.01)</td>
</tr>
<tr>
<td>&gt;0–3 cups/d</td>
<td>794</td>
<td>668</td>
<td>126</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>&gt;3–6 cups/d</td>
<td>1542</td>
<td>1275</td>
<td>267</td>
<td>1.11 (0.88, 1.40)</td>
<td>0.83 (0.64, 1.07)</td>
</tr>
<tr>
<td>&gt;6 cups/d</td>
<td>390</td>
<td>331</td>
<td>59</td>
<td>0.95 (0.68, 1.32)</td>
<td>0.67 (0.46, 0.98)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 cups/d</td>
<td>231</td>
<td>214</td>
<td>17</td>
<td>0.38 (0.23, 0.64)</td>
<td>0.54 (0.31, 0.92)</td>
</tr>
<tr>
<td>&gt;0–3 cups/d</td>
<td>1173</td>
<td>970</td>
<td>203</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>&gt;3–6 cups/d</td>
<td>2737</td>
<td>2203</td>
<td>534</td>
<td>1.16 (0.97, 1.38)</td>
<td>0.93 (0.76, 1.12)</td>
</tr>
<tr>
<td>&gt;6 cups/d</td>
<td>1048</td>
<td>846</td>
<td>202</td>
<td>1.14 (0.92, 1.42)</td>
<td>0.83 (0.65, 1.07)</td>
</tr>
</tbody>
</table>

1 Hypertension was defined as having at least stage 1 hypertension (mean systolic blood pressure over both follow-up measurements ≥140 mm Hg, mean diastolic blood pressure over both follow-up measurements ≥90 mm Hg, or the use of antihypertensive medication at any of both follow-up measurements) (14). Normotensive subjects at baseline were defined as having a systolic blood pressure <140 mm Hg and a diastolic blood pressure <90 mmHg and use of no antihypertensive medication. Odds ratios (ORs) were obtained from logistic regression with persistent hypertension (yes or no) as the dependent variable and dummy categories of coffee intake and adjustment factors as independent variables. The category of >0–3 cups coffee/d was the reference group. The interaction between sex and coffee intake in relation to hypertension was borderline statistically significant (P = 0.08). Among coffee drinking women, there was a statistically significant trend over coffee intake categories (P = 0.023).

2 Adjusted for baseline age, height and weight, smoking, alcohol intake, tea intake, education level, occupational status, and total energy intake. For the analysis of the total cohort, sex was added to the adjustment model.

3 The OR was statistically significant.

Our study indicates that both abstainers from coffee and, in women only, heavy consumers (>6 cups/d) have lower risks of hypertension than do low coffee consumers (>0–3 cups/d). An association between higher coffee consumption and lower blood pressure seems to be present only after middle age.

Nonresponses may have been related to coffee intake or blood pressure, but not likely to specific associations between coffee and blood pressure. We cannot rule out that professional advice to lower coffee consumption to persons with higher baseline intakes has led to a spurious association between higher intake and lower blood pressure. However, we analyzed persons who were normotensive at baseline to whom such advice was unlikely given. Although there may be residual or unmeasured confounding, we accounted for most confounders that are known to be risk factors for high blood pressure. We had no baseline information about intake of caffeine-containing sodas, which was recently shown to be associated with incidence of hypertension (7). We can only speculate about the effects of adjustment for that intake, but consumption of such sodas in the Netherlands in middle-aged persons in that period was probably low. A strong feature of our study is that it pertains to a large sample from the general population with >10 y of follow-up. Moreover, our outcome classification—the incidence of hypertension persisting over a 5-y period—better reflects true hypertension incidence than does measurement at a single occasion. This outcome was based on actual blood pressure measurements or hypertension treatment,
Among normotensives at baseline, we showed a lower hypertension risk between caffeinated coffee intake and hypertension risk (7). Further, an inverse U-shaped association was found between back coffee intake and cardiovascular morbidity or mortality (14). The question is how low coffee intake is associated with a higher risk of hypertension, rather than on self-reporting of a physician diagnosis, such as in the Nurse’s Health Study (7). Finally, although we have addressed a limited number of hypotheses concerning the association between coffee intake and hypertension, we cannot rule out chance as an explanation, neither for the main effects nor for the interactions with age.

Our findings in men agree with those of a previous report on a long-term follow-up conducted in 1017 males showing a nonsignificant relation between coffee intake and hypertension incidence, despite a small positive effect on blood pressure levels (6). Our findings in women may be compatible with recent findings in the women participating in the Nurse’s Health Studies I and II, where an inverse U-shaped association was found between caffeine intake and hypertension risk (7). Among normotensives at baseline, we showed a lower hypertension risk for coffee abstainers than for users of >0–3 cups/d.

From short-term randomized trials, it is known that coffee and caffeine have a blood pressure–raising effect (1). The prevailing explanation for such effect is that caffeine antagonizes endogenous adenosine, resulting in vasoconstriction and elevated total peripheral vascular resistance (15). The question is how low coffee intake is associated with a higher risk of hypertension, whereas high coffee intake is not. One explanation could be that effects of coffee on blood pressure do depend on consumption habits, with higher blood pressure levels observed in nonhabitual than in habitual coffee consumers (16, 17). Thus, a higher hypertension risk in consumers of >0–3 cups/d than in nondrinkers and consumers of >6 cups/d may be based on a lower adaptation to the effects of coffee when used in moderate to low amounts. Alternatively, coffee may have more general protective effects, because our finding of heavier coffee intake leading to lower blood pressure does not seem to be specific. A lower risk for type 2 diabetes mellitus in heavy coffee users compared with non- or moderate users was first shown by one of us (18) and recently confirmed by others (19-23) and is somewhat stronger in women than in men (21). In agreement with our findings for hypertension, it was shown that women with low coffee use (<1 cup/d) had a higher risk of type 2 diabetes mellitus than did non-coffee users (20). However, it remains unclear whether and to what extent an association between coffee intake and blood pressure can explain the association with diabetes mellitus. Some studies could only adjust for known hypertension (18, 23), which may be misclassified and leave residual confounding by blood pressure as an explanation. One study adjusted for systolic blood pressure levels, whereas diastolic blood pressure levels were most strongly associated with diabetes mellitus risk (21). In other studies, there was no explicit adjustment for blood pressure or hypertension (19, 20, 22). Finally, there may be a blood pressure–lowering compound in coffee that explains lower hypertension risk with higher intake. It was recently shown in a cross-sectional study that higher habitual tea intake was associated with lower risk of hypertension (24). This effect of tea combined with our observations on coffee may indicate a central role of serum potassium concentrations. A higher intake of potassium is clearly associated with lower blood pressure (25), and tea and particularly coffee are rich sources of potassium in Western diets (26). This, however, would not explain the observed lower hypertension risk in coffee abstainers.

Overall, higher baseline coffee intake in our study was associated with lower blood pressure only from middle age onwards, whereas there was no such relation in younger persons. We can only speculate about the mechanisms for such age-dependent effects. It may agree with the results from short-term randomized trials, in which blood pressure–raising effects of coffee were reported to be stronger in younger than in older subjects (1) and which may point at more habituation to coffee in younger persons (15, 16). Alternatively, if there is a protective salt constituent in coffee, such as potassium, it may be through increasing salt-sensitivity and higher blood pressure levels observed with increasing age (27, 28) that the protective effects of higher coffee intake become apparent. Finally, there is recent evidence to suggest that genetically determined slow caffeine metabolism in relation to cardiovascular disease risk is present only in relatively younger persons (29).

From a public health point of view, a direct implication of our study may be to reduce the incidence of hypertension by measures aimed at refraining from moderate coffee intake, but that would be unpractical if at all effective. The most important merit of our study is the elucidation of the role of coffee intake through its relation with hypertension in increasing the risk of cardiovascular disease. Although there are reports claiming coffee to be hazardous (30, 31), the larger cohorts show no association between coffee intake and cardiovascular morbidity or mortality (32) or with the prognosis of myocardial infarction (33). We consider it likely that the extent to which coffee intake explains
hypertension risk is too small to be detected in relation to cardiovascular disease. A practical implication from our findings would therefore be to abstain from professional advice concerning coffee intake in normotensive individuals, which indeed agrees with the latest clinical guidelines on hypertension (14). We cannot preclude that associations between coffee intake and cardiovascular outcomes are different among hypertensive individuals (34). In conclusion, coffee abstinence was associated with a lower hypertension risk than was low coffee consumption, and an inverse U-shaped relation between coffee intake and risk of hypertension was observed in women.

We thank the epidemiologists and fieldworkers of the Municipal Health Service in Doetinchem for their contribution to the data collection for this study. We thank A Bloksstra, PE Steinberger, and AWD van Kessel for data management, J Steenbrink-van Woerden and P Vissink for logistic support, and E van der Wolf for secretarial assistance.

WMMV was the project leader. WMMV, HBBdM, and MO were involved in the design and conduct of the cohort. CSPMU, HCB, and DEG analyzed the data. All authors played a role in data-interpretation and writing of the manuscript. CSPMU was provided an unrestricted grant by the organization on Physiological Effects of Coffee (PEC) in Paris. No other authors had any conflicts of interest.

REFERENCES


Effects of a reduced-glycemic-load diet on body weight, body composition, and cardiovascular disease risk markers in overweight and obese adults1–3

Kevin C Maki, Tia M Rains, Valerie N Kaden, Kathleen R Raneri, and Michael H Davidson

ABSTRACT

Background: Lowering the dietary glycemic load and increasing protein intake may be advantageous for weight management.

Objective: This randomized controlled trial was designed to evaluate the effects of an ad libitum reduced-glycemic-load (RGL) diet on body weight, body composition, and cardiovascular disease (CVD) risk markers in overweight and obese adults during an initial weight-loss phase (12 wk) and a weight-loss maintenance phase (weeks 24–36).

Design: Subjects were assigned to RGL (n = 43) or low-fat, portion-controlled (control; n = 43) diet groups. The RGL group was instructed to eat until satisfied, maintaining a low-carbohydrate intake during weeks 0–2 and adding low-glycemic-index carbohydrate thereafter. Control subjects were instructed to reduce fat intake and decrease portion sizes, with a targeted energy deficit of 500 to 800 kcal/d.

Results: The RGL group had lost significantly more weight than did the control group at week 12 (−4.9 and −2.5 kg, respectively; P = 0.002), but the 2 groups did not differ significantly at week 36 (−4.5 and −2.6 kg, respectively; P = 0.085). Changes in fat mass differed between the groups at week 12 (−1.9 and −0.9 kg, respectively; P = 0.016) but not at week 36 (−2.0 and −1.3 kg, respectively; P = 0.333). At the end of the study, no differences were found in responses for CVD risk markers except a larger mean change in HDL cholesterol in the RGL group than in the control group (3.8 and 1.9 mg/dL, respectively; P = 0.037).

Conclusion: These findings provide evidence that an ad libitum RGL diet is a reasonable alternative to a low-fat, portion-controlled eating plan for weight management.

KEY WORDS Glycemic load, obesity, weight loss, body composition, cardiovascular disease risk markers, glucose tolerance, randomized controlled trial

INTRODUCTION

The prevalence of obesity in the United States has more than doubled during the past 25 y (1). Recent estimates from population-based samples suggest that nearly two-thirds of adults in the United States are overweight or obese (2). This fact is generating considerable public health concern because excess adiposity is associated with a greater risk of the development of diabetes mellitus, atherosclerotic cardiovascular disease (CVD), and several forms of cancer (1).
weight-loss period and at the end of a weight-maintenance period at week 36. Secondary outcomes included changes in body composition, selected CVD risk markers, and health-related quality of life at weeks 12 and 36.

SUBJECTS AND METHODS

Subjects

Potential participants were recruited from the Chicago metropolitan area and screened by telephone. Eligibility was further assessed at a screening visit. To qualify for entry into the study, men and women had to be aged 18–65 y, have a waist circumference measurement at week −1 of ≥87 cm for women and ≥90 cm for men, and be judged by the investigators to be in good health on the basis of medical history and routine laboratory tests. Subjects had to be willing to discontinue all use of dietary supplements or multivitamins, except those provided during the study, and to follow the assigned diet and maintain their usual level of physical activity throughout the trial.

Volunteers were excluded from participation if they had experienced a weight loss of >4.5 kg in the 2 mo before screening, had a body mass index (BMI; in kg/m²) ≥37.0, were current smokers (any cigarette use), or had a history of smoking in the 6 mo before screening. Subjects were also excluded from participation if they had diabetes mellitus, uncontrolled hypertension, or a history of cancer (other than successfully resected basal cell carcinoma) in the past 2 y.

Subjects with a history of or current significant cardiac, renal, pulmonary, hepatic, biliary, or endocrine disease were excluded, as were those with a history of recurrent nephrolithiasis or acute nephrolithiasis within the year before screening. Subjects were excluded from participation if they had used any weight-loss medication, supplements, programs, or meal-replacement products intended to alter body weight during the 4 wk before screening or if they had any diagnosis of eating disorder, a history of surgery for weight-reducing purposes, or a clinically significant gastrointestinal disorder.

Additional exclusion criteria included the use of systemic corticosteroids, androgens, phenytoin, or pseudoephedrine; lipid-lowering therapies (unless dose-stable for 2 mo before enrollment); drugs for regulating hemostasis other than dose-stable aspirin; thyroid hormones (except stable-dose replacement therapy) for ≥2 mo before enrollment); or psychiatric medications.

Postmenopausal women who were current users of sex hormone therapy or who had discontinued use in the 2 mo before screening were excluded. Female subjects who were pregnant, planning to be pregnant during the study period, or lactating or those of childbearing potential who were not using an approved method of contraception were also excluded.

This trial was performed according to Good Clinical Practice Guidelines, the Declaration of Helsinki (2000), and US 21 CFR Part 50 – Protection of Human Subjects, and Part 56 – Institutional Review Boards. An institutional review board, Quorum (Seattle, WA), approved the protocol and the informed consent document before the initiation of the study. Study procedures were reviewed with subjects, and each participant provided written informed consent and authorization for the release of protected health information before study procedures were carried out.

Clinic visits

This trial used a randomized, controlled design with 2 parallel treatment arms. Subjects were randomly assigned to either the RGL or portion-controlled (control) diet. Weeks 0–12 were weight-loss treatment. At some point between weeks 12 and 24, each subject transitioned to a weight-maintenance phase. From week 24 on, all subjects were in the weight-maintenance phase.

The study included 15 clinic visits: 1 screening visit (week −1), 1 visit at baseline (week 0), 7 visits during the weight-loss treatment phase (weeks 1, 2, 4, 6, 8, 10, and 12), 3 visits during the transition from weight-loss treatment to weight-loss maintenance (weeks 16, 20, and 24), and 3 visits during the weight-loss maintenance phase (weeks 28, 32, and 36).

Visits were conducted between 0700 am and 1200. Subjects were asked to refrain from consuming any foods or beverages except water for ≥10 h and to abstain from consuming alcohol for ≥24 h before visits.

At week −1, subjects provided written informed consent and completed a medical history questionnaire. Concomitant medications used 8 wk before and during the study were recorded at this and subsequent visits. Body weight, height, and waist circumference were measured, and vital signs were assessed. Samples were taken for serum chemistry, hematology, lipid panel, and urinalysis.

At the randomization (baseline) visit (week 0), subjects received diet instruction and information on their assigned diets for them to take home. All subjects were provided with daily multivitamins (Centrum; Wyeth Consumer Healthcare, Madison, NJ) and were instructed to bring back any unused multivitamins at each subsequent study visit.

At each visit, vital signs were assessed and anthropometric measurements were taken, daily food checklists were reviewed and used in dietary counseling or reinforcement, and any concomitant use of medication and adverse events were assessed. Fasting serum lipid values were obtained at weeks −1, 0, 10, 12, 32, and 36. Serum chemistry, hematology, urinalysis, and fasting insulin and glucose values were measured at weeks −1, 12, and 36. Dual-energy X-ray absorptiometry (DXA) was conducted at weeks 0, 12, 24, and 36 for assessment of body composition. Three-day diet records were analyzed by using the NUTRIENT DATA SYSTEM FOR RESEARCH (version 4.06; University of Minnesota, Minneapolis, MN) at weeks 0, 2, 6, 12, 24, and 36. The Willett Food-Frequency Questionnaire was completed at weeks 0, 12, and 36 for estimation of dietary GI and GL (20). The questionnaire uses published sources of GI values for carbohydrates from specific foods for calculation of the weighted average GI of the diet (20). GI is calculated as the weighted average GI multiplied by the total daily carbohydrate intake (20).

Laboratory measurements

The dietitians test (Ketostix; Bayer Diagnostics, Tarrytown, NY) was used to assess urinary ketone concentrations. Other laboratory measurements, including serum chemistry, hematology, insulin, urinalysis, and lipid concentrations, were conducted by Medical Research Laboratories (MRL, Highland Heights, KY). Serum chemistry analysis (including fasting glucose) was completed with a chemistry analyzer (Hitachi 747–200; Roche Diagnostics Corporation, Indianapolis, IN), and hematologic testing was conducted with the use of a complete count analyzer (Coulter STKS; Coulter Corporation, Miami, FL).
Cholesterol and triacylglycerol were measured enzymatically by using the Hitachi 747–200. Heparin and manganese chloride were used to precipitate apolipoprotein-containing particles to allow measurement of HDL cholesterol. LDL cholesterol in mg/dL was calculated by using the Friedewald equation: LDL cholesterol = (total cholesterol – HDL cholesterol – triacylglycerol)/5 (21). The homeostasis model assessment (HOMA) of insulin resistance was calculated as glucose (mmol/L) × insulin (mU/L)/22.5 (22).

Blood pressure

Blood pressure was obtained after the subject had been sitting quietly for 5 min. Systolic and diastolic pressures were measured with a standard manual mercury sphygmomanometer. Two measurements, separated by 2 min, were taken and averaged. If the measurements differed by >5 mm Hg, an additional reading was obtained, and all 3 readings were averaged.

Body composition and anthropometric measurements

Whole-body DXA scans were performed (QDR 4500A; Hologic Inc, Waltham, MA). Body composition values (fat mass and fat-free mass) were measured with the use of Hologic SYSTEMS Software (version 9.03D; Hologic Inc) according to the procedures outlined in the Hologic QDR 4500 user’s guide of 1995.

Anthropometric measurements included subjects’ height (first visit only), weight, and waist circumference. At each visit, the measurement of waist circumference was performed at the level of the iliac crest by using a nonstretchy anthropometric tape measure. Two measurements were taken and averaged. If these differed by >0.5 cm, a third measurement was taken and the outlying value was discarded.

Diet implementation

Diet counselors were the same for both groups. Diet training manuals were created to standardize training of the counselors, all of whom were Registered Dietitians with extensive experience in counseling for weight management. For both diet groups, handouts were provided to each subject for at-home use, and dietary guidance was reinforced at each treatment visit.

For subjects assigned to the control diet, energy needs for weight maintenance were estimated from basal EE calculated with the Harris-Benedict equation (23) multiplied by an activity factor of 1.2, 1.3, or 1.4 after evaluation of activity level from the results from the Stanford 7-d physical activity questionnaire (24). EE values obtained at weeks 12 and 36 were adjusted (multiplied by 1.24) to account for the 2 comparisons (26). This adjustment was used to obtain statistical significance.

Those following the RGL diet were instructed to eat 3 meals/d plus snacks and to eat until hunger was satisfied. Specific high-carbohydrate foods, including all starchy foods and fruits, were eliminated during phase 1 of the diet (the first 2 wk). Subjects were advised not to consume any alcohol during this period and were instructed on selection of foods with low carbohydrate content that are not high in saturated and trans fats to avoid excessive intake of cholesterol-raising fatty acids. At week 2, subjects received instructions for the second phase of the diet (weeks 2–12), during which low-GI foods, such as high-fiber fruits and vegetables, beans, and whole-grain breads and cereals, were restored to the diet. Moderate alcohol consumption was allowed after week 2.

At week 12, both groups were given the option to remain in the weight-loss phase of the diet or switch to the weight-loss maintenance phase. Subjects who opted to continue in the weight-loss phase at week 12 were given the option to switch at each subsequent visit until week 24 to accommodate individual weight-loss goals. At week 24, all subjects who had not yet done so were asked to switch to weight-loss maintenance. During the weight-maintenance phase, subjects were advised to continue the portion-controlled or RGL diet while eating sufficient quantities of food to maintain the level of body weight achieved at the time of switching from weight loss to weight maintenance.

Assessment of quality of life

A quality-of-life instrument was completed by all subjects at weeks 0, 12, 24, and 36 (SF-36 Health Survey 2002; Medical Outcomes Trust and QualityMetric Inc, Lincoln, RI).

Statistical analysis

Statistical analyses were conducted with SAS software (versions 8.02 and 9.1; SAS Institute, Cary, NC). All tests of statistical significance were performed at alpha = 0.05, 2-sided, unless otherwise indicated. The Shapiro-Wilk test (25) was used to test variables for normality. Where necessary because of non-normality, rank transformations were employed before calculation of inferential statistics. Baseline comparability of treatment groups was assessed by 1-factor analysis of variance (ANOVA) for continuous variables and chi-square or Fisher’s exact test for categorical variables.

An intent-to-treat analysis of outcome variables was completed by using all data for subjects with ≥1 postrandomization body weight measurement. For this analysis, which was considered primary, the last nonbaseline observation was carried forward for missing data points. A secondary analysis for body weight was also completed, in which the baseline weight was substituted after discontinuation for all subjects who dropped out during the treatment period. In addition, analyses were completed that excluded subjects who did not complete the 36-wk study or who violated the protocol in some material way. However, because results from these analyses did not differ materially from the results from the intent-to-treat analyses, only the latter are presented.

For all continuous variables, repeated-measures ANOVA models were employed for values at baseline and weeks 2 (dietary variables only), 12, and 36. These models each included terms for treatment, time, and treatment × time interaction. Pairwise comparisons between groups were completed for individual timepoints when the treatment × time interaction term was significant (P ≤ 0.05) in the repeated-measures model. Body weight responses at weeks 12 and 36 were considered the primary outcome variables and were assessed by ANOVA, with baseline body weight and treatment group as factors in the model. For both the primary and secondary body weight response analyses, the P values obtained at weeks 12 and 36 were adjusted (multiplied by 1.724) to account for the 2 comparisons (26). This adjustment was used to obtain statistical significance.
In addition, previously specified analyses of changes from baseline to week 12 (end of the initial weight-loss period) and week 36 (end of the weight-maintenance period) were completed for body composition, anthropometric, and laboratory variables by using 1-factor ANOVA with treatment as a fixed effect. Exploratory analyses were completed to assess maximal weight loss and the changes from the point of maximal weight loss to week 36 in both treatment groups. Multiple linear regression analysis was used to explore determinants of the changes in body composition. For all secondary outcome and exploratory variables, unadjusted P values are presented.

Safety analyses included all subjects who were randomly assigned. Safety and tolerability were assessed by evaluation of adverse events that occurred during treatment, laboratory test results, and vital signs measurements. Adverse events were coded by using the World Health Organization dictionary. Fisher’s exact test was used to test for differences between groups in adverse events overall and for each body system and coded term.

RESULTS

Subjects

A summary of subject disposition is shown in Figure 1. A total of 122 men and women were screened for entry into the trial, 86 of whom qualified and were randomly assigned (n = 43/group). No significant differences were observed between groups in the percentage of subjects who participated in the study through weeks 12 (81.4% in each group; P = 1.00) and 36 (53.5% in the RGL group and 69.8% in the control group; P = 0.120), respectively.

Baseline characteristics of the study sample are shown in Table 1. Subjects were approximately two-thirds female. Fifty-two percent classified themselves as non-Hispanic white and 35% as African American. The mean age of the participants was 50 y; mean BMI was 32, and 67% of the subjects were obese (BMI ≥30). The fasting insulin concentration tended to be higher among subjects in the RGL group than among those in the control group (10.3 and 9.0 mU/L, respectively; P = 0.061). Otherwise, the groups were well matched with regard to baseline characteristics.
One subject in each group dropped out of the trial after randomization but before providing any individual data for body weight or composition. These subjects were excluded from the intent-to-treat analyses.

**Body weight and composition**

At the end of the initial 12-wk weight-loss period, the mean weight change was $-4.9 \pm 0.5$ kg in the RGL arm and $-2.5 \pm 0.5$ kg in the control arm (adjusted $P = 0.002$), as shown in Figure 2. At week 12, 24 subjects (55%) in the RGL group and 9 subjects (21%) in the control group had achieved a loss of $\geq 5\%$ of body weight ($P = 0.002$). An exploratory analysis showed that mean maximum weight loss was significantly ($P = 0.005$) larger in the RGL group (6.4 $\pm$ 0.6 kg) than in the control group (4.4 $\pm$ 0.6 kg).

By the end of the weight-maintenance period (at week 36), the mean weight change was $-4.5 \pm 0.7$ kg in the RGL group and $-2.6 \pm 0.9$ kg in the control group (adjusted $P = 0.085$), as shown in Figure 2. At week 36, 45% and 29% of subjects in the RGL and control groups, respectively, were $\geq 5\%$ below their baseline body weights ($P = 0.114$). An exploratory analysis of weight change from the point of maximal weight loss to week 36 found that the groups did not differ significantly in the mean amount of weight regained during the weight-maintenance period (1.9 $\pm$ 0.3 kg in the RGL group and 1.8 $\pm$ 0.3 kg in the control group; $P = 0.866$).

In the secondary analysis, in which the baseline body weight was substituted for missing values after subject dropout, the change from baseline to week 12 differed between groups, with significantly ($P = 0.018$) greater weight loss apparent in the RGL group than in the control group ($-4.9 \pm 0.6$ and $-3.0 \pm 0.6$ kg, respectively). However, no significant difference between groups was present at week 36 ($-2.8 \pm 0.7$ and $-2.2 \pm 0.8$ kg for the RGL and control groups, respectively; $P = 0.684$).

As shown in Table 2, body fat and fat-free mass were lower in both groups at week 12, and the reductions in the RGL arm were significantly ($P = 0.016$ and $< 0.001$ for body fat and fat-free mass, respectively) greater in the RGL arm than in the control arm. A significant ($P = 0.004$) difference between groups in the loss of fat-free mass persisted at 36 wk, whereas changes in fat mass from baseline were no longer significantly different ($-2.0$ and $-1.3$ kg for the RGL and control groups, respectively; $P = 0.333$). No significant difference in the loss of fat-free mass at week 12 was seen between groups after adjustment for body weight loss by using multiple linear regression ($P = 0.162$). However, RGL treatment was associated with significantly ($P = 0.037$) greater loss of fat-free mass at week 36, after adjustment for the difference in body weight response, than was the control diet. Waist circumference also declined in both groups, but the difference in response between groups was not significant at week 12 ($P = 0.082$) or week 36 ($P = 0.783$).

**Physical activity and dietary composition**

No significant differences between groups were observed in physical activity at baseline (Table 1) or at any timepoint during treatment (data not shown). Estimates of dietary composition

---

**TABLE 1**

Baseline characteristics by treatment group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RGL group ($n = 43$)</th>
<th>Control group ($n = 43$)</th>
<th>$P^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex [n (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>29 (67.4)</td>
<td>29 (67.4)</td>
<td>1.000</td>
</tr>
<tr>
<td>Men</td>
<td>14 (32.6)</td>
<td>14 (32.6)</td>
<td></td>
</tr>
<tr>
<td>Ethnicity [n (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic white</td>
<td>19 (44.2)</td>
<td>26 (60.5)</td>
<td>0.129</td>
</tr>
<tr>
<td>African American</td>
<td>15 (34.9)</td>
<td>15 (34.9)</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>6 (14.0)</td>
<td>1 (2.3)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>3 (7.0)</td>
<td>1 (2.3)</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>47.9 $\pm$ 1.8$^c$</td>
<td>51.4 $\pm$ 1.5</td>
<td>0.142</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>91.2 $\pm$ 2.0</td>
<td>88.7 $\pm$ 1.8</td>
<td>0.353</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>32.1 $\pm$ 0.6</td>
<td>31.6 $\pm$ 0.5</td>
<td>0.580</td>
</tr>
<tr>
<td>Physical activity (MET-h/wk)</td>
<td>312.2 $\pm$ 14</td>
<td>309.8 $\pm$ 10.0</td>
<td>0.672</td>
</tr>
</tbody>
</table>

$^1$ RGL, reduced glycemic load; MET, metabolic equivalent.
$^2$ $P$ for continuous variables generated from ANOVA with treatment as a factor and for categorical variables from $2 \times 2$ chi-square tests (ethnicity was classified as non-Hispanic white or other).
$^c$ $\bar{x} \pm$ SEM (all such values).

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**FIGURE 2.** Least-squares mean ($\pm$SEM) changes in body weight from baseline by treatment group assignment: ◆, control group ($n = 42$); ■, reduced-glycemic-load group ($n = 42$). The last available nonbaseline value was carried forward to impute missing values. Significant effects were present for time and treatment $\times$ time interaction ($P < 0.001$ for both) in an initial repeated-measures ANOVA model. $P$ values shown in the figure were generated from ANOVA models with baseline body weight and treatment group as factors; they were multiplied by 1.724 to adjust for 2 previously specified endpoint comparisons (weeks 12 and 36).
from analysis of 3-d food records and Willett Food-Frequency Questionnaires (for GI and load) are shown in Table 3. The groups did not differ significantly at baseline in any variable except alcohol intake, which was higher in the RGL group than in the control group (6 and 2 g/d, respectively; P = 0.010).

Reported energy consumption was reduced from baseline in both groups throughout the treatment period. Differences between treatment arms did not reach significance, although mean values were 4.7% to 10.5% lower in the RGL group than in the control group at each timepoint after randomization. Consistent values were 4.7% to 10.5% lower in the RGL group than in the control group at all timepoints during treatment, but the treatment \\
time interaction was not significant (P = 0.270).

Mean GI in the RGL group declined from 52 at baseline to 46 at week 12 and 48 at week 36 (P ≤ 0.001 versus control group at both timepoints) and remained essentially unchanged from baseline in the control group (52, 51, and 51 at baseline, week 12, and week 36, respectively). The combination of lower GI and reduced carbohydrate consumption produced reductions of 43% to 49% in mean GI in the RGL group but reductions of 12% to 16% in the control group (P ≤ 0.002 at weeks 12 and 36).

Alcohol was not allowed during the first 2 wk of the RGL treatment plan, which resulted in a significant difference in intake between groups at week 2 (P < 0.001), but intake did not differ at weeks 12 and 36. Reported protein intake was higher in the RGL group at all timepoints during treatment, but the treatment \\
time interaction was not significant (P = 0.067). Intakes of total, saturated, and unsaturated fats were also higher in the RGL group than in the control group, but differences did not reach significance. Mean dietary cholesterol intake was higher (122–259 mg/d) in the RGL group than in the control group at all timepoints during the treatment (P < 0.001).

Dietary fiber, expressed in g/1000 kcal, did not differ between groups. No significant differences between groups were observed in calcium, magnesium, potassium, or sodium intakes during treatment.

Cardiovascular disease risk markers, ketones, and quality of life

Mean values for CVD risk markers by group at baseline and changes from baseline are shown in Table 4. No significant differences between groups were present at baseline. The only significant difference in lipid responses between the RGL and control diet groups was in the increase in HDL cholesterol at week 36 (3.8 and 1.9 mg/dL, respectively; P = 0.037).

The RGL dietary regimen was designed to have sufficient carbohydrate to prevent significant ketonemia, even during the initial period of more severe carbohydrate restriction. At baseline, 4 subjects—1 in the RGL group and 3 in the control group—had measurable concentrations of urinary ketones (P = 0.270). At week 2, 12 subjects (29%) in the RGL group and 1 subject (3%) in the control group had urinary ketones (P = 0.001). Of the 12 subjects with urinary ketones in the RGL group, 6 were classified as having trace amounts and 6 as having small-to-moderate concentrations. At weeks 12 (14.6% in the RGL group and 7.1% in the control group; P = 0.273) and 36 (12.2% in the RGL group and 2.4% in the control group; P = 0.084), the prevalence of subjects with urinary ketones in the 2 groups did not differ significantly, but it tended to be higher in the RGL group.

No differences between groups at baseline or subsequent timepoints were observed for any domain in the SF-36 quality-of-life questionnaire (data not shown).

DISCUSSION

This randomized, controlled trial showed that an ad libitum RGL diet produced significantly greater losses of body weight and fat during an initial weight-loss period than did a traditional,
### TABLE 3
Dietary composition, including dietary supplements, from analyses of 3-d diet records and Willett food-frequency questionnaires by timepoint and treatment group

<table>
<thead>
<tr>
<th>Variable</th>
<th>RGL group</th>
<th>Control group</th>
<th>Time$^2$</th>
<th>Treatment $\times$ time$^2$</th>
<th>Pairwise$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy (kcal/d)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42 2050 ± 131$^d$</td>
<td>42 1961 ± 132</td>
<td>&lt;0.001</td>
<td>0.555</td>
<td>0.651</td>
</tr>
<tr>
<td>Week 2</td>
<td>41 1365 ± 72</td>
<td>41 1525 ± 87</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Week 12</td>
<td>41 1343 ± 54</td>
<td>41 1500 ± 107</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Week 36</td>
<td>41 1533 ± 78</td>
<td>41 1608 ± 113</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Carbohydrate (g/d)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42 222 ± 18</td>
<td>42 218 ± 17</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.811</td>
</tr>
<tr>
<td>Week 2</td>
<td>41 69 ± 6</td>
<td>41 168 ± 12</td>
<td>—</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Week 12</td>
<td>41 108 ± 6</td>
<td>41 171 ± 14</td>
<td>—</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Week 36</td>
<td>41 131 ± 9</td>
<td>41 186 ± 15</td>
<td>—</td>
<td>—</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Sucrose + fructose (g/d)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42 64 ± 8</td>
<td>42 58 ± 6</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.696</td>
</tr>
<tr>
<td>Week 2</td>
<td>41 17 ± 2</td>
<td>41 44 ± 6</td>
<td>—</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Week 12</td>
<td>41 25 ± 3</td>
<td>41 46 ± 6</td>
<td>—</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Week 36</td>
<td>41 28 ± 2</td>
<td>41 51 ± 7</td>
<td>—</td>
<td>—</td>
<td>0.005</td>
</tr>
<tr>
<td><strong>Glycemic index$^c$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42 52 ± 1</td>
<td>42 52 ± 1</td>
<td>&lt;0.001</td>
<td>—</td>
<td>0.799</td>
</tr>
<tr>
<td>Week 12</td>
<td>38 46 ± 1</td>
<td>35 51 ± 1</td>
<td>&lt;0.001</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Week 36</td>
<td>38 48 ± 1</td>
<td>36 51 ± 1</td>
<td>—</td>
<td>—</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Glycemic load$^c$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42 14355 ± 1146</td>
<td>42 14409 ± 1148</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.974</td>
</tr>
<tr>
<td>Week 12</td>
<td>38 7298 ± 867</td>
<td>35 12649 ± 1060</td>
<td>—</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Week 36</td>
<td>38 8173 ± 816</td>
<td>36 12118 ± 836</td>
<td>—</td>
<td>—</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Alcohol (g/d)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42 6 ± 2</td>
<td>42 3 ± 1</td>
<td>0.021</td>
<td>0.006</td>
<td>0.010</td>
</tr>
<tr>
<td>Week 2</td>
<td>41 0 ± 0</td>
<td>41 3 ± 1</td>
<td>—</td>
<td>—</td>
<td>0.001</td>
</tr>
<tr>
<td>Week 12</td>
<td>41 3 ± 1</td>
<td>41 3 ± 1</td>
<td>—</td>
<td>—</td>
<td>0.907</td>
</tr>
<tr>
<td>Week 36</td>
<td>41 5 ± 1</td>
<td>41 5 ± 2</td>
<td>—</td>
<td>—</td>
<td>0.572</td>
</tr>
<tr>
<td><strong>Protein (g/d)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42 80 ± 4</td>
<td>42 82 ± 5</td>
<td>0.065</td>
<td>0.067</td>
<td>0.832</td>
</tr>
<tr>
<td>Week 2</td>
<td>41 97 ± 5</td>
<td>41 75 ± 3</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Week 12</td>
<td>41 87 ± 4</td>
<td>41 70 ± 3</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Week 36</td>
<td>41 87 ± 4</td>
<td>41 76 ± 5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Total fat (g/d)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42 92 ± 7</td>
<td>42 85 ± 8</td>
<td>&lt;0.001</td>
<td>0.305</td>
<td>0.355</td>
</tr>
<tr>
<td>Week 2</td>
<td>41 80 ± 6</td>
<td>41 62 ± 5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Week 12</td>
<td>41 63 ± 4</td>
<td>41 61 ± 6</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Week 36</td>
<td>41 72 ± 5</td>
<td>41 63 ± 6</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td><strong>Saturated fat (g/d)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42 31 ± 3</td>
<td>42 28 ± 3</td>
<td>&lt;0.001</td>
<td>0.325</td>
<td>0.294</td>
</tr>
<tr>
<td>Week 2</td>
<td>41 24 ± 2</td>
<td>41 19 ± 2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Week 12</td>
<td>41 20 ± 2</td>
<td>41 19 ± 2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Week 36</td>
<td>41 23 ± 2</td>
<td>41 18 ± 2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Unsaturated fat (g/d)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42 54 ± 4</td>
<td>42 51 ± 5</td>
<td>&lt;0.001</td>
<td>0.327</td>
<td>0.552</td>
</tr>
<tr>
<td>Week 2</td>
<td>41 49 ± 4</td>
<td>41 38 ± 3</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Week 12</td>
<td>41 38 ± 2</td>
<td>41 37 ± 3</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Week 36</td>
<td>41 43 ± 3</td>
<td>41 40 ± 4</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Cholesterol (mg/d)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42 317 ± 26</td>
<td>42 273 ± 24</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.266</td>
</tr>
<tr>
<td>Week 2</td>
<td>41 487 ± 45</td>
<td>41 228 ± 19</td>
<td>—</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Week 12</td>
<td>41 347 ± 27</td>
<td>41 221 ± 20</td>
<td>—</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Week 36</td>
<td>41 339 ± 23</td>
<td>41 217 ± 20</td>
<td>—</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Dietary fiber (g · d$^{-1}$ · 1000 kcal$^{-1}$)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42 8 ± 1</td>
<td>42 9 ± 1</td>
<td>0.050</td>
<td>0.393</td>
<td>0.456</td>
</tr>
<tr>
<td>Week 2</td>
<td>41 11 ± 1</td>
<td>41 12 ± 1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Week 12</td>
<td>41 13 ± 1</td>
<td>41 12 ± 1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Week 36</td>
<td>41 11 ± 1</td>
<td>41 12 ± 1</td>
<td>—</td>
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<td>—</td>
</tr>
<tr>
<td><strong>Calcium (mg/d)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42 715 ± 69</td>
<td>42 724 ± 74</td>
<td>0.056</td>
<td>0.453</td>
<td>0.979</td>
</tr>
<tr>
<td>Week 2</td>
<td>41 886 ± 60</td>
<td>41 783 ± 41</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Week 12</td>
<td>41 824 ± 45</td>
<td>41 793 ± 55</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Week 36</td>
<td>41 838 ± 51</td>
<td>41 839 ± 54</td>
<td>—</td>
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</tr>
</tbody>
</table>
interact to influence appetite. An RGL diet is likely to produce a lower average daylong insulin concentration (3, 28–30). This is especially true for persons with insulin resistance and compensatory hyperinsulinemia, both of which are characteristic features of obesity (31–33). In persons with excess adiposity, the ability of insulin to promote glycogenesis is impaired to a greater extent than is its ability to promote glucose oxidation (31). In an insulin-resistant person, a high-GL diet would be expected to produce extended periods of hyperinsulinemia that would, in turn, increase whole-body carbohydrate oxidation and depress fat oxidation (34). Because it has been shown that increased hepatic fat oxidation is associated with lower hunger and food intake in animals and humans (7, 35–37), the reduced fat oxidation associated with hyperinsulinemia may lead to an increase in food intake.

Protein consumption tended to be higher in the RGL group than in the control group. Protein appears to have greater ability than does carbohydrate to promote satiety and reduce subsequent food intake (38–40). In addition, numerous studies have shown elevated thermic responses to meals and higher 24-h EE with increased protein intake (40). Diets with higher protein content have also been found to produce greater weight loss in some trials, which may be accounted for by these effects (40–42).

Previous studies showed that 20–30% of weight lost by dieting is typically fat-free mass (43). In this study, loss of fat-free mass was significantly larger in the RGL group at week 36 (∼51% and 41% of weight lost in the RGL and control groups, respectively), even after statistical adjustment for total weight loss (P = 0.037). The greater reduction in fat-free mass is of potential concern, because this reduction could result in attenuation of total daily EE if attributable to a greater loss of lean tissue (44). Because DXA cannot distinguish between changes in non-bone lean tissue and those in fluid, the degree to which fluid loss may have contributed to the loss of fat-free mass is unclear.

Participants transitioned from the initial weight-loss period to a weight-maintenance phase by week 24. Subjects in both groups regained a portion of the maximum amount of weight lost: 1.9 kg and 1.8 kg in the RGL and control diets, respectively. At the end of the weight-maintenance period (week 36), differences between groups losses of body weight and fat were no longer significant. In addition, no significant differences were observed in CVD risk markers, except for a larger increase in HDL cholesterol at week 36 in subjects in the RGL group than in the control group (3.8 and 1.9 mg/dL, respectively; P < 0.037). These findings suggest that, whereas the RGL diet produced greater initial losses of body weight and fat than did the portion-controlled diet, no evidence was observed of any adverse effects on CVD risk markers.

The differential losses of body weight and fat during the weight-loss phase indicate that subjects in the RGL group experienced greater negative energy balance than did those in the control group. This difference must be explained by greater EE, lower energy intake, or some combination of these factors. The decline in resting EE during weight loss may be attenuated with an RGL diet (3, 5). Rats and mice fed an RGL diet, achieved by use of a low-GI chow (made with amylose), required more energy to maintain the same body weight than did animals fed high-GI chow (made with amylopectin) (27). Despite higher energy intake, animals fed the RGL diet had lower adiposity after 9–18 wk than did those fed the control diet (27). These findings support the view that an RGL diet may affect EE and partitioning in ways that would favor negative energy balance.

Dietary GL, insulin secretion, and substrate oxidation may interact to influence appetite. An RGL diet is likely to produce a lower average daylong insulin concentration (3, 28–30). This is especially true for persons with insulin resistance and compensatory hyperinsulinemia, both of which are characteristic features of obesity (31–33). In persons with excess adiposity, the ability of insulin to promote glycogenesis is impaired to a greater extent than is its ability to promote glucose oxidation (31). In an insulin-resistant person, a high-GL diet would be expected to produce extended periods of hyperinsulinemia that would, in turn, increase whole-body carbohydrate oxidation and depress fat oxidation (34). Because it has been shown that increased hepatic fat oxidation is associated with lower hunger and food intake in animals and humans (7, 35–37), the reduced fat oxidation associated with hyperinsulinemia may lead to an increase in food intake.

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### Table 3 (Continued)

<table>
<thead>
<tr>
<th>Variable</th>
<th>RGL group</th>
<th>Control group</th>
<th>Time</th>
<th>Treatment × time</th>
<th>Pairwise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium (mg/d)</td>
<td>n</td>
<td>n</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>42</td>
<td>42</td>
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<tr>
<td>Week 2</td>
<td>41</td>
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<tr>
<td>Week 12</td>
<td>41</td>
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<tr>
<td>Week 36</td>
<td>41</td>
<td>41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td></td>
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<td>0.001</td>
<td>0.207</td>
<td>0.583</td>
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<tr>
<td>Treatment × time</td>
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<tr>
<td>Pairwise</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 RGL, reduced glycemic load. Results for all variables except glycemic index and glycemic load are from analyses of 3-d food records; glycemic index and glycemic load results were derived from analyses of Willett food-frequency questionnaires.

2 P values from repeated-measures ANOVA models with treatment, time, and treatment × time interaction as factors.

3 P values from ANOVA models comparing treatments at each timepoint.

4 ± SEM (all such values).

5 Calculated as the weighted average glycemic index value for all carbohydrate-containing foods in the diet.

6 Calculated as each food’s carbohydrate quantity (in g) multiplied by the respective glycemic index.
Because insulin stimulates renal sodium and water reabsorption, an RGL diet may be expected to produce fluid loss (10, 45–47). However, insulin is also a growth factor that enhances protein synthesis and inhibits protein catabolism in muscle tissues (48, 49), and thus some greater loss of lean tissue may also have occurred if total circulating insulin exposure was reduced. Additional research will be needed to differentiate between changes in the masses of fluid and those in lean tissue with consumption of RGL diets.

A common concern expressed regarding carbohydrate-restricted diets is that they may be associated with higher consumption of saturated fatty acids and cholesterol, which could lead to higher LDL cholesterol. In a meta-analysis comparing low-carbohydrate with low-fat weight-loss diets, Nordmann et al (19) found that both HDL (4.6 mg/dL) and LDL (5.4 mg/dL) cholesterol were higher after 6 mo in subjects assigned to the low-carbohydrate diet. In the current study, reported saturated fat and cholesterol intakes were

<table>
<thead>
<tr>
<th>Variable</th>
<th>RGL group</th>
<th>Control group</th>
<th>Time</th>
<th>Treatment × time</th>
<th>Pairwise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>42</td>
<td>199.3 ± 4.5</td>
<td>42</td>
<td>206.5 ± 6.5</td>
<td>&lt;0.001</td>
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<tr>
<td>Δ at week 12</td>
<td>39</td>
<td>−12.2 ± 2.7</td>
<td>38</td>
<td>−8.3 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>Δ at week 36</td>
<td>39</td>
<td>−1.5 ± 3.9</td>
<td>38</td>
<td>−3.0 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42</td>
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<td>123.4 ± 5.7</td>
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<td>−7.0 ± 2.2</td>
<td>38</td>
<td>−3.6 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Δ at week 36</td>
<td>39</td>
<td>−2.8 ± 3.2</td>
<td>38</td>
<td>−1.9 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42</td>
<td>56.2 ± 2.0</td>
<td>42</td>
<td>56.4 ± 2.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Δ at week 12</td>
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<td>−0.2 ± 1.2</td>
<td>38</td>
<td>−2.1 ± 0.9</td>
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</tr>
<tr>
<td>Δ at week 36</td>
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<td>3.8 ± 1.4</td>
<td>38</td>
<td>1.9 ± 0.8</td>
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<td>Triacylglycerol (mg/dL)</td>
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<td></td>
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<tr>
<td>Baseline</td>
<td>42</td>
<td>127.1 ± 8.3</td>
<td>42</td>
<td>134.0 ± 10.6</td>
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<tr>
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<td>−24.8 ± 5.3</td>
<td>38</td>
<td>−11.5 ± 6.5</td>
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</tr>
<tr>
<td>Δ at week 36</td>
<td>39</td>
<td>−12.5 ± 5.2</td>
<td>38</td>
<td>−15.5 ± 8.9</td>
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<tr>
<td>Glucose (mg/dL)</td>
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<td></td>
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<td>Baseline</td>
<td>43</td>
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<td>95.2 ± 1.7</td>
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<td>−1.1 ± 1.7</td>
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<td>2.6 ± 1.4</td>
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<td>Insulin (mU/L)</td>
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<td></td>
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<tr>
<td>Baseline</td>
<td>43</td>
<td>10.4 ± 1.0</td>
<td>43</td>
<td>9.0 ± 1.2</td>
<td>0.003</td>
</tr>
<tr>
<td>Δ at week 12</td>
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<td>−0.4 ± 1.1</td>
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<td>0.9 ± 1.1</td>
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</tr>
<tr>
<td>Δ at week 36</td>
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<td>1.1 ± 1.0</td>
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<td>2.4 ± 0.7</td>
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<tr>
<td>HOMA insulin resistance</td>
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<td>Baseline</td>
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<td>2.4 ± 0.2</td>
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<td>2.1 ± 0.3</td>
<td>0.003</td>
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<tr>
<td>Δ at week 12</td>
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<td>−0.2 ± 0.3</td>
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<tr>
<td>Δ at week 36</td>
<td>39</td>
<td>0.3 ± 0.3</td>
<td>39</td>
<td>0.7 ± 0.2</td>
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<td>114.7 ± 1.6</td>
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<td>Δ at week 12</td>
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<td>−1.2 ± 2.3</td>
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</tr>
<tr>
<td>Δ at week 36</td>
<td>42</td>
<td>0.2 ± 1.7</td>
<td>42</td>
<td>0.1 ± 2.2</td>
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<tr>
<td>Diastolic blood pressure (mm Hg)</td>
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<td>Δ at week 12</td>
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<td>−0.6 ± 1.4</td>
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</tr>
<tr>
<td>Δ at week 36</td>
<td>42</td>
<td>−4.1 ± 1.8</td>
<td>42</td>
<td>−1.6 ± 1.3</td>
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</tr>
</tbody>
</table>

7 RGL, reduced glycemic load; HOMA, homeostasis model assessment.

P values from repeated-measures ANOVA models comparing treatments at each timepoint.

Average of values obtained at weeks 1 and 0.

Average of values obtained at weeks 10 and 12.

Average of values obtained at weeks 32 and 36.
higher in the RGL diet group than in the control group. Whereas the increase in HDL cholesterol at 36 wk was greater in the RGL diet group than in the control group (3.8 and 1.9 mg/dL, respectively; \( P = 0.037 \)), a finding that is consistent with the results of the meta-analysis, no difference was found between the groups in the mean change in LDL cholesterol (−2.8 and −1.9 mg/dL, respectively; \( P = 0.836 \)).

Jenkins et al (50) showed that, under conditions of identically matched food intake, a lower rate of carbohydrate absorption, elicited by consumption of 17 snacks as opposed to 3 larger meals, was associated with a 28% lower average insulin concentration and a mean reduction of 13.5% in LDL-cholesterol concentrations. Sloth et al (14) also found that ad libitum consumption of a low-GI diet resulted in a 10% decrease in LDL cholesterol, whereas consumption of a high-GI diet matched for macronutrient and fiber content per 1000 kcal resulted in a slight (2%) rise in LDL cholesterol.

Insulin has a stimulatory effect on VLDL synthesis (32). Therefore, a smaller integrated insulin response may reduce VLDL synthesis and entry into the circulation (5, 51). In the absence of changes in the rates of VLDL conversion to LDL or of LDL removal from the circulation (or both), a reduction in the concentration of LDL cholesterol would be expected. However, greater intakes of saturated fats and cholesterol can suppress hepatic LDL uptake (52), which may explain the absence of a larger reduction in LDL cholesterol in subjects consuming the RGL diet than in those consuming the control diet (53).

In summary, the results of the current trial showed that, in free-living, overweight and obese subjects, an ad libitum RGL diet produced greater losses of body weight and fat than did a traditional, portion-controlled diet during an initial weight-loss period. Weight regain from the point of maximal weight loss did not differ between treatments. However, the differences in body weight and body fat responses between groups were no longer significant at the end of the weight-maintenance phase of the trial (week 36). No evidence was present of any adverse effects of the RGL diet group.

In conclusion, the results of the current study suggest that an ad libitum RGL diet is a reasonable alternative to a low-fat, portion-controlled weight-loss diet. Additional research is warranted to clarify the mechanisms responsible for the greater initial losses of body weight and fat associ-ated with the RGL diet, to evaluate the persistence of those losses over longer treatment periods, and to obtain greater insight into strategies that would improve long-term weight-loss mainte-nance.

The authors thank Denise Umporowicz, Kimberly Oldham, and Marjorie Bell for assistance with data management and statistical analyses. All authors were responsible for the design of the study; KCM was responsible for the statistical analyses; all authors contributed to the final interpretation of the data; KCM wrote the draft of the manuscript, and all authors participated in revising the manuscript. TMR and KRR were employees of Kraft Foods at the time the trial was conducted. None of the other authors had any personal or financial conflict of interest.

REFERENCES
27. Pawlik DB, Kushner JA, Ludwig DS. Effects of dietary glycemic index


42. Layman DK. Protein quantity and quality at levels above the RDA improves adult weight loss. J Am Coll Nutr 2004;23:631S–6S.


47. Scott CL. Diagnosis, prevention, and intervention for the metabolic syndrome. Am J Cardiol 2003;92:35i–42iS.


Soy inclusion in the diet improves features of the metabolic syndrome: a randomized crossover study in postmenopausal women

Leila Azadbakht, Masoud Kimiagar, Yadollah Mehrabi, Ahmad Esmailzadeh, Mojgan Padyab, Frank B Hu, and Walter C Willett

ABSTRACT

Background: Little evidence exists regarding the effects of soy consumption on the metabolic syndrome in humans.

Objective: We aimed to determine the effects of soy consumption on components of the metabolic syndrome, plasma lipids, lipoproteins, insulin resistance, and glycemic control in postmenopausal women with the metabolic syndrome.

Design: This randomized crossover clinical trial was undertaken in 42 postmenopausal women with the metabolic syndrome. Participants were randomly assigned to consume a control diet (Dietary Approaches to Stop Hypertension, DASH), a soy-protein diet, or a soy-nut diet, each for 8 wk. Red meat in the DASH period was replaced by soy-protein in the soy-protein period and by soy-nut in the soy-nut period.

Results: The soy-nut regimen decreased the homeostasis model of assessment-insulin resistance score significantly compared with the soy-protein (difference in percentage change: −7.4 ± 0.8; P < 0.01) or control (−12.9 ± 0.9; P < 0.01) diets. Consumption of soy-nut also reduced fasting plasma glucose more significantly than did the soy-protein (−5.3 ± 0.5%; P < 0.01) or control (−5.1 ± 0.6%; P < 0.01) diet. The soy-nut regimen decreased LDL cholesterol more than did the soy-protein period (−5.0 ± 0.6%; P < 0.01) and the control (−9.5 ± 0.6%; P < 0.01) diet. Soy-nut consumption significantly reduced serum C-peptide concentrations compared with control diet (−8.0 ± 2.1; P < 0.01), but consumption of soy-protein did not.

Conclusion: Short-term soy-nut consumption improved glycemic control and lipid profiles in postmenopausal women with the metabolic syndrome. Am J Clin Nutr 2007;85:735−41.

KEY WORDS Metabolic syndrome, insulin resistance, soy, glycemic control, lipid profiles

INTRODUCTION

The metabolic syndrome is a clustering of metabolic abnormalities that occurs in individuals with impaired insulin sensitivity (1, 2). Existing data suggest that the incidence of the metabolic syndrome is rising at an alarming rate (3, 4). In Tehran, Iran, it has been estimated to affect >30% of adults (5), a prevalence significantly higher than that of most developed countries (6). The etiology of this syndrome is largely unknown: genetic, metabolic, and environmental factors, including diet, are thought to play a major role (7). In previous investigations, intakes of unsaturated fatty acids (8), n−3 fatty acids (9), dairy products (10), and whole grains (11) appeared to influence the prevalence of this syndrome, either positively or negatively, but little emphasis has been placed on the specific therapeutic diets that control the metabolic syndrome. In choosing a therapeutic diet, high amounts of vegetables, fruits, legumes, whole grains, low-fat dairy foods, and low amounts of saturated fat and salt have been used in previous studies (12−15). Foods that improve insulin sensitivity might also modulate the metabolic abnormalities linked with insulin resistance (16).

Many studies have reported beneficial effects of soy consumption on human health, but most of these investigations have been conducted among type 2 diabetic or hypercholesterolemic patients (16−19), or healthy subjects (20). Although some studies have reported effects of soy consumption on the metabolic syndrome in animals (21, 22), to our knowledge no reports are available regarding the effects of soy intake on features of the metabolic syndrome in humans. Soy consumption could reduce the risk of the metabolic syndrome through its beneficial components, including complex carbohydrates, unsaturated fatty acids, vegetable protein, soluble fiber, oligosaccharides, vitamins, minerals, inositol-derived substances such as lipintol and pinitol, and phytoestrogens, particularly the isoflavones genistein, diadzein, and glycitein (23−28). However, the amount and kind of these components may vary in different kinds of soy products; ie, textured soy-protein or soy-nut. We evaluated the effects of soy consumption (in the form of soy-protein and unsalted soy-nut) on features of the metabolic syndrome, including plasma lipids, lipoproteins, insulin resistance, and glycemic control, in postmenopausal women with the metabolic syndrome.

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SUBJECTS AND METHODS

Participants

A total of 120 postmenopausal women were screened for inclusion in the study. The study was conducted in Tehran in 2005. Women were considered postmenopausal if menstrual periods had been absent for > 1 y and follicle-stimulating hormone, serum luteinizing hormone, testosterone, and estradiol concentrations confirmed their status (29). The metabolic syndrome was defined according to Adult Treatment Panel (ATP) III guidelines (30): 1) abdominal adiposity (waist circumference > 88 cm); 2) low concentrations of serum HDL cholesterol (< 50 mg/dL); 3) hypertriglyceridemia (≥ 150 mg/dL); 4) elevated blood pressure (≥ 130 mm Hg systolic blood pressure and ≥ 85 mm Hg diastolic blood pressure); and 5) impaired glucose homeostasis (≥ 110 mg/dL). To be enrolled in the study, patients had to have ≥ 3 of the above-mentioned criteria to be classified as having metabolic syndrome. Exclusion criteria were any secondary cause of hyperglycemia, current or previous (in the preceding 6 mo) use of estrogen therapy, treatment with insulin or oral hypoglycemic agents, untreated hypothyroidism, smoking, kidney or liver diseases, and breast malignancy or breast cancer. Finally, a total of 42 postmenopausal women who had 5 components of the metabolic syndrome and met the inclusion criteria were included in the present study. All participants provided informed written consent. The present study was approved by the research council and ethical committee of the National Nutrition and Food Technology Research Institute of Shaheed Beheshti University of Medical Sciences.

Study procedures

We used a randomized crossover design. After 3 wk of run-in on a usual diet (55% of energy from carbohydrate, 15% of energy from protein, and 30% of energy from fat), we randomly assigned women to consume the control diet (Diet A = red meat-DASH diet), DASH diet with soy-nut (Diet B = soy-nut period), or DASH diet with soy-protein (Diet C = soy-protein period) each for 8 wk. Each patient received all 3 diets and had 2 washout periods (each washout for 4 wk) between the 3 diets. We set 6 different sequence of diet intakes (ABC, ACB, BCA, BAC, CBA, and CAB) with 2 washouts in each model; patients randomly followed one of these sequences. The randomization was conducted at the end of the run-in.

Measurements were obtained before run-in, after run-in, after each diet, and after each washout. Baseline measurements were performed at the beginning of each dietary period. The participants were free-living during the study period and they prepared their own meals. Only soy products were given to them during the different phases of the study. The participants were asked not to change their habitual physical activity level for the duration of the study. The patients recorded their physical activities for 3 days each month.

Diets

We used 3 diets. 1) Control diet: this diet was a DASH diet. The general recommendation for macronutrient composition of the DASH diet was the following: 50–60% of energy as carbohydrate, 15–20% of energy as protein, and < 30% of energy as total fat. This diet had one serving of red meat (red meat-DASH) and was rich in fruit, vegetables, whole grains, and low-fat dairy products and was low in saturated fat, total fat, cholesterol, refined grains, and sweets. The sodium intake was 2400 mg/d (31). Sodium intake was determined according to the Iranian Food Composition Table. We prescribed little added salt during cooking (only 1 tsp) and no table salt. 2) Diet with soy-nut: this diet was the same as the control diet, but we replaced red meat with soy-nut. Every 30 g soy-nut was considered 1 serving of red meat (32). 3) Diet with soy-protein: this diet was the same as the control diet, but we replaced red meat with soy-protein. Every 30 g soy-protein was considered 1 serving of red meat (32). Soy-nut was produced by Toos manufacturer in Mashhad, Iran, and soy-protein has produced by Sobhan manufacturer in Behshahr, Iran. The nutrient compositions of the soy-nut and soy-protein consumed by the study participants, based on our analysis, are shown in Table 1. Soy-protein composition data was determined by biochemical analysis. No significant differences were found between the results of the analysis and those that were calculated with the food-composition tables. The amount of soy isoflavones consumed was 84 mg/d (8 mg genistein, 43 mg diadzein, and 33 mg genestein) during the soy-protein period and 102 mg/d (9 mg genistein, 53 mg genestein, and 40 mg diadzein) during the soy-nut period. The patients received education in how they could prepare their meals with soy-protein. A nutritionist explained to the participants how to wash the soy-protein products, soak them for 30 min, and then cook them in boiling water with turmeric, lemon juice, and tomato paste for 10 min.

The calorie requirements of each participant were calculated individually on the basis of equations suggested by the Institute of Medicine, Food and Nutrition Board (33). Weight loss was not a goal, and patients were not on a weight-reducing diet. The participants were visited every 2 wk for 45–60 min per patient. They were in touch with the study nutritionist daily by phone. For measuring food intake, 3-d diet records were used at baseline and during the intervention for each month. Every participant had to bring her 3-d diet record and physical activity records every month when they were reviewed by the study staff; these records were used for checking diet compliance. Each food and beverage in the diet records was then coded according to the prescribed protocol and analyzed for content of energy and the other nutrients by using NUTRITIONIST III software (version 7.0; N-Squared Computing, Salem, OR), which was designed for Iranian foods. Physical activity records were activities in MET-h/d.

The study nutritionist explained the benefits of each diet for patients. Patients also received education in using an exchange list of foods and in writing food diaries. The diets were individually prescribed by using a calorie count system, and an exchange

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Soy protein</th>
<th>Soy nut</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g)</td>
<td>15</td>
<td>11.3</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>0.3</td>
<td>7</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Total phytoestrogens (mg)</td>
<td>84</td>
<td>102</td>
</tr>
<tr>
<td>Glycitein (mg)</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Genistein (mg)</td>
<td>43</td>
<td>53</td>
</tr>
<tr>
<td>Diadzein (mg)</td>
<td>33</td>
<td>40</td>
</tr>
</tbody>
</table>

*Amounts are per 30 g.*
list was given to each patient for exchanging the food items and calculating calories. A 7-d menu cycle at 6-calorie levels (1800, 1900, 2000, 2100, 2200, and 2300 calories) was developed for each diet. Subjects were free-living and they provided their own meals. To maximize treatment fidelity, group discussions, in which the food items that should be eaten were emphasized, were performed monthly. Patients also received education on the methods of preparing soy-protein according to their menu and were encouraged to follow their diets. The investigators randomly took part in the counseling sessions and controlled the messages that nutritionist was giving to each group. Patient adherence was assessed by analyzing the 3-d food record diaries monthly and by the attendance at the meetings and monthly visits.

**Measurements**

Body weight was measured while the subjects were minimally clothed without shoes by using digital scales and recorded to the nearest 0.1 kg. Height was measured in a standing position, without shoes, by using a tape meter while the shoulders were in a normal state. Waist circumference (WC) was measured to the nearest 0.1 cm at the narrowest level over light clothing, with the use of an unstretched tape measure, without any pressure to body surface. Blood pressure was measured twice after the participants sat for 15 min.

Twelve-hour fasting blood samples were collected into tubes containing 0.1% EDTA and were centrifuged at 4 °C and 500 × g for 10 min to separate the plasma. Blood glucose was measured on the day of blood collection by an enzymatic colorimetric method by using glucose oxidase. Serum total cholesterol and triacylglycerol concentration were measured with commercially available enzymatic reagents (Pars Azmoon, Tehran, Iran) adapted to a Selectra autoanalyzer (Vital Scientific, Spankeren, Netherlands). HDL cholesterol was measured after precipitation of the apolipoprotein B–containing lipoproteins with phosphotungstic acids (34). Inter-and intraassay CVs were both <5% for all measurements. Serum insulin concentrations were measured by using an enzyme-linked immunosorbent assay (ELISA; Diagnostic Biochem Canada Inc, Montreal, Canada). The CV, which was calculated by using duplicate study samples, was <10%; the analytic sensitivity was 2 μU/mL. Insulin resistance was calculated by using the homeostasis model of assessment-insulin resistance (HOMA-IR) (35). Serum luteinizing hormone and follicle-stimulating hormone were measured by radioimmunoassay; testosterone and estradiol were measured by ELISA. C-peptide was also measured by ELISA (Diagnostic Biochem Canada Inc) (36). Plasma phytoestrogens were measured by HPLC according to Franke et al (37, 38). Serum concentrations of apolipoprotein B 100 (apoB-100) and apoA-I were determined by ELISA (Diagnostic Biochem Canada Inc). We used standard and control solutions for all measurements, all laboratory measurements were standardized, and standard curves were plotted for all of them. Soy-derived phytoestrogens were measured by HPLC to determine the patients’ adherence (38).

**Statistical analysis**

We used 3 methods of analyses to be confident that the results were similar. First, we used general linear models to compare the means of the metabolic variables at the end of the soy-nut, soy-protein, and control phases. Then, we used Tukey’s test as a post-hoc test for comparing the end of treatment values of each group with each other group. The percentage change for each variable was also calculated by the formula \[\frac{(E - B) \times 100}{B}\], where \(E\) is the end of treatment value and \(B\) is the baseline value. We compared groups using the percentage change in both repeated-measures analysis of variance and paired \(t\) test analyses. We also determined the mean percentage change differences, which were derived by calculating the differences in percentage change for each variable in pair-wise group comparisons. This parameter gives the most direct estimate of the difference in response in comparing groups. Interactions between soy intake and weight were not significant for any of the metabolic features. Period and treatment order effects were tested by using the appropriate general linear models.

For skewed variables (apoA-I and fasting insulin concentration), we used log-transformed values in all analyses and reported the geometric means. Pearson correlation coefficients were used to evaluate the relation between soy-derived phytoestrogens intake (calculated from self reported soy intake in 3-d diet records) and plasma phytoestrogen concentrations. All results were considered significant if the two-tailed \(P\) value was < 0.05. Statistical analysis was performed by using SPSS for WINDOWS version 13.0 (SPSS, Chicago, IL) and SAS version 8.2 (SAS Institute Inc, Cary, NC).

**RESULTS**

All participants (42 postmenopausal women with the metabolic syndrome) completed the entire crossover study. Calculated nutrient content and food group servings of 3-d diet records according to the patients’ report is shown in Table 2. Both the soy-nut and soy-protein diets were well tolerated. Only one person complained of feeling bloated during the soy-protein period. The activity level of the subjects remained the same across all study periods \([\bar{x} (±SE)\) physical activity in control period: 2.38 ± 0.19 MET-h/d; in soy-protein period: 2.50 ± 0.26 MET-h/d; and in soy-nut period: 2.44 ± 0.26; \(P = 0.10)\]

The baseline characteristics of the study participants and the effects of the 3 diets on components of the metabolic syndrome, lipid profiles, and glycemic control are shown in Table 3. No significant differences in the baseline characteristics of the study participants were seen. Significant differences between the end values of control diet, soy-protein regimen, and soy-nut consumption for glycemic control indexes were seen. A post-hoc comparison of the diets showed a significant difference between the control and soy-protein diets with regard to LDL cholesterol, total cholesterol, fasting insulin, HOMA-IR, and apoB-100. Similar results were seen for fasting plasma glucose (FPG), LDL cholesterol, total cholesterol, fasting insulin, HOMA-IR, C-peptide, and apoB-100 in a comparison of the soy-nut and control diets. No significant effects of period or treatment order were observed. Compared with the control diet, plasma phytoestrogen increased significantly after the soy-nut regimen (percentage change: 64%; \(P < 0.01)\) and after the soy-protein diet (percentage change: 48%; \(P < 0.01)\).

**DISCUSSION**

We found that soy as a replacement for red meat in a DASH eating plan had beneficial effects on features of the metabolic syndrome, soy-nut being more effective than soy-protein. We
also found that soy consumption improved glycemic control and cardiovascular disease risk factors, at least in the short term, in postmenopausal women with the metabolic syndrome. Although some studies have assessed the effects of soy intake on the metabolic syndrome in animals (21, 22), to our knowledge this is the first study in which such an effect has been evaluated in humans.

Both soy-nut and soy-protein had beneficial effects on serum concentrations of total cholesterol, LDL cholesterol, triacylglycerol, and apoB-100. Such results have also been seen among subjects with different types of diseases (39, 40). Beneficial effects of soy consumption on blood lipids were the most consistently reported findings. In a meta-analysis of 38 controlled clinical trials, Anderson et al (18) showed significant reductions in total cholesterol (9%), LDL cholesterol (13%), and triacylglycerols (11%) with the consumption, on average, of 47 g soy-protein/d. Two recent meta-analyses concluded that the isoflavone content of soy may be responsible for its lipid-lowering effect (41, 42). Controversy still exists in the field regarding the relative contribution of potential mechanisms of action of soy-protein, isoflavones, and other soy components on blood lipids and lipoproteins.

Besides abnormalities in lipid metabolism, elevated blood pressure is another feature of the metabolic syndrome. Most studies showed no effect on blood pressure with consumption of soy-protein containing isoflavones (25, 43, 44). In the current study, neither soy-protein nor soy-nut consumption had significant effects on systolic or diastolic blood pressures compared with the control diet. It seems that the blood pressure-lowering effect of the overall DASH diet in the 3 periods of the study may be responsible for the changes in blood pressure during the soy-nut or soy-protein periods.

We also observed that soy consumption improved glycemic control. HOMA-IR decreased significantly at the end of the soy-nut diet compared with the soy-protein and control diets. This finding may support a direct pharmacologic effect of soy constituents. The hypothesis that soy isoflavones modulate glycemic control is not proven yet. We assumed that a higher level of pinitol, and protein might have beneficial effects on glycemic control. HOMA-IR decreased significantly at the end of the soy-nut diet compared with the soy-protein and control diets. This finding may support a direct pharmacologic effect of soy constituents. The hypothesis that soy isoflavones modulate glycemic control is not proven yet. We assumed that a higher level of pinitol, and protein might have beneficial effects on glycemic control.

In most studies, the phytoestrogens, amino acids, and fatty acid content of soy-nuts were suggested to be responsible for its lipid-lowering effect (16, 45–47). In the present study, neither soy-protein nor soy-nut consumption had significant effects on systolic or diastolic blood pressures compared with the control diet. It seems that the blood pressure-lowering effect of the overall DASH diet in the 3 periods of the study may be responsible for the changes in blood pressure during the soy-nut or soy-protein periods.

Differences between the 3 trial periods (repeated-measures ANOVA).

In the current study, neither soy-protein nor soy-nut consumption had significant effects on systolic or diastolic blood pressures compared with the control diet. It seems that the blood pressure-lowering effect of the overall DASH diet in the 3 periods of the study may be responsible for the changes in blood pressure during the soy-nut or soy-protein periods.
Table 3: Features of the metabolic syndrome at baseline and after 8 wk of intervention in postmenopausal women

<table>
<thead>
<tr>
<th>Metabolic variables</th>
<th>Control(^1)</th>
<th>Soy protein(^2)</th>
<th>Soy nut(^3)</th>
<th>Overall P(^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight(^6)</td>
<td>70.0 ± 0.9</td>
<td>70.0 ± 0.8</td>
<td>70.1 ± 0.8</td>
<td>0.98</td>
</tr>
<tr>
<td>End of trial</td>
<td>70.1 ± 0.9</td>
<td>70.7 ± 0.9</td>
<td>70.4 ± 0.8</td>
<td>0.57</td>
</tr>
<tr>
<td>Waist (cm)(^6)</td>
<td>91.5 ± 0.7</td>
<td>91.4 ± 0.7</td>
<td>91.2 ± 0.8</td>
<td>0.60</td>
</tr>
<tr>
<td>End of trial</td>
<td>91.9 ± 0.8</td>
<td>91.5 ± 0.9</td>
<td>91.0 ± 1.0</td>
<td>0.19</td>
</tr>
<tr>
<td>SBP (mm Hg)(^6)</td>
<td>136 ± 0.7</td>
<td>136 ± 0.7</td>
<td>136 ± 0.7</td>
<td>0.97</td>
</tr>
<tr>
<td>End of trial</td>
<td>131 ± 1.2</td>
<td>132 ± 0.7</td>
<td>131 ± 1.0</td>
<td>0.26</td>
</tr>
<tr>
<td>DBP (mm Hg)(^6)</td>
<td>87 ± 0.1</td>
<td>87 ± 0.2</td>
<td>87 ± 0.2</td>
<td>0.16</td>
</tr>
<tr>
<td>End of trial</td>
<td>84.0 ± 0.5</td>
<td>85.0 ± 0.5</td>
<td>85.0 ± 0.5</td>
<td>0.85</td>
</tr>
<tr>
<td>Triacylglycerols (mg/dL)(^6)</td>
<td>120 ± 0.6</td>
<td>119 ± 0.6</td>
<td>118 ± 0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>End of trial</td>
<td>112 ± 1.0a</td>
<td>111 ± 0.9a</td>
<td>103 ± 0.5b</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)(^6)</td>
<td>31.0 ± 0.4</td>
<td>32.0 ± 0.4</td>
<td>32.0 ± 0.4</td>
<td>0.91</td>
</tr>
<tr>
<td>End of trial</td>
<td>33.3 ± 0.7</td>
<td>34.0 ± 0.7</td>
<td>33.3 ± 0.4</td>
<td>0.51</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)(^6)</td>
<td>143 ± 0.8</td>
<td>142 ± 0.6</td>
<td>137 ± 3.2</td>
<td>0.08</td>
</tr>
<tr>
<td>End of trial</td>
<td>134 ± 3.3a</td>
<td>127 ± 2.4a</td>
<td>118 ± 3.0c</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)(^6)</td>
<td>238 ± 1.0</td>
<td>239 ± 0.9</td>
<td>238 ± 0.9</td>
<td>0.37</td>
</tr>
<tr>
<td>End of trial</td>
<td>228 ± 0.9a</td>
<td>217 ± 0.5a</td>
<td>209 ± 0.6c</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fasting insulin (μIU/mL)(^7)</td>
<td>14.3 ± 0.09</td>
<td>14.2 ± 0.09</td>
<td>14.1 ± 0.09</td>
<td>0.73</td>
</tr>
<tr>
<td>End of trial</td>
<td>14.2 ± 0.09a</td>
<td>13.3 ± 0.04b</td>
<td>12.8 ± 0.09c</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HOMA-IR(^8)</td>
<td>4.19 ± 0.03</td>
<td>4.20 ± 0.04</td>
<td>4.16 ± 0.06</td>
<td>0.65</td>
</tr>
<tr>
<td>End of trial</td>
<td>3.9 ± 0.04a</td>
<td>3.6 ± 0.03b</td>
<td>3.3 ± 0.03c</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C-peptide (ng/mL)(^6)</td>
<td>2.09 ± 0.04</td>
<td>2.1 ± 0.03</td>
<td>2.1 ± 0.02</td>
<td>0.79</td>
</tr>
<tr>
<td>End of trial</td>
<td>1.92 ± 0.04a</td>
<td>1.86 ± 0.03a</td>
<td>1.77 ± 0.04b</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Apolipoprotein AI (g/L)(^7)</td>
<td>1.33 ± 0.02</td>
<td>1.30 ± 0.02</td>
<td>1.32 ± 0.01</td>
<td>0.51</td>
</tr>
<tr>
<td>End of trial</td>
<td>1.32 ± 0.01</td>
<td>1.30 ± 0.01</td>
<td>1.31 ± 0.01</td>
<td>0.35</td>
</tr>
<tr>
<td>Apolipoprotein B100 (g/L)(^6)</td>
<td>1.33 ± 0.02</td>
<td>1.28 ± 0.01</td>
<td>1.25 ± 0.01</td>
<td>0.61</td>
</tr>
<tr>
<td>End of trial</td>
<td>1.08 ± 0.03a</td>
<td>0.98 ± 0.03b</td>
<td>0.92 ± 0.04b</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

\(^1\) n = 42. SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting blood glucose; HOMA-IR, homeostasis model of assessment-insulin resistance. Means with different superscript letters are significantly different, P < 0.05 (Tukey’s test). No significant period effect or interaction of period effect and treatment effect were observed for any of the variables.

\(^2\) This diet provided one serving of red meat and was rich in fruit, vegetables, whole grains, and low-fat dairy products and was low in saturated fat, total fat, cholesterol, refined grains, and sweets. The sodium intake was 2400 mg/d [Dietary Approach to Stop Hypertension (DASH) pattern].

\(^3\) This diet was the same as the control diet (DASH diet), but we replaced red meat with soy-nut. Every 30 g soy-nut was considered 1 serving of red meat.

\(^4\) This diet was the same as the control diet (DASH diet), but we replaced red meat with soy-protein. Every 30 g soy-protein was considered 1 serving of red meat.

\(^5\) Comparison of the 3 diet periods (repeated-measures ANOVA).

\(^6\) Values are \(\bar{x} \pm \text{SE}\).

\(^7\) Values are geometric \(\bar{x} \pm \text{SE}\).

The amount of soy isoflavones consumed was 84 mg/d during the soy-protein period and 102 mg/d during the soy-nut period. This is lower than the doses used in previous effective trials (16, 40), but it is higher than the isoflavone content of diets commonly consumed in some Asian countries where soy is a staple food (20–80 mg/d) (47). In some situations, excessive soy-protein intake could do more harm than good; some evidence suggests that genistein can stimulate estrogen-receptor positive breast cancers to grow (50). We excluded any patients with breast malignancy or breast cancer from the present study, and we used textured soy-protein or soy-nut, which were food sources of isoflavones, rather than pure isoflavones or soy-protein pills.
The washout period of 4 wk between 2 treatment phases in our study seemed adequate, because the values of metabolic risk factors returned to baseline levels before the start of the next trial. A strength of the present study was the good compliance of our subjects, which was confirmed by the phytoestrogen concentrations in each period of the study. We did not evaluate the effects of soy-protein or soy-nut according to estrogen receptor genotype in our participants; in some studies, responses to isoflavone consumption have varied according to estrogen receptor genotype (51–53). Also, further studies may be warranted to assess the effect of soy consumption on features of the metabolic syndrome while taking the “equol producer” or “equol nonproducer” status into account (51–53). In conclusion, our findings suggest that short-term soy-nut consumption may reduce insulin resistance and improve glycemic control and lipid concentrations in postmenopausal women with the metabolic syndrome.

We thank the participants of this study for their enthusiastic support. We would also like to thank Amy Cohen from Harvard School of Public Health for her valuable help.

LA and AE designed the study, collected and analyzed the data, and wrote the manuscript. MK served as a supervisor and YM as advisor for this research. MP helped with the statistical analysis. FBH and WCW commented on this work and helped in the manuscript preparation. None of the authors have any personal or financial conflicts of interest.

REFERENCES

Evaluation of a portable device to measure daily energy expenditure in free-living adults

Maxime St-Onge, Diane Mignault, David B Allison, and Rémi Rabasa-Lhoret

ABSTRACT

Background: Increasing daily energy expenditure (EE) plays an important role in the prevention or treatment of several lifestyle-related diseases; however, its measurement remains problematic.

Objective: The objective was to evaluate a portable armband device for measuring daily and physical activity EE compared with doubly labeled water (DLW) in free-living individuals.

Design: Daily EE and physical activity EE were measured in 45 subjects over a 10-d period simultaneously with 2 techniques: a portable armband and DLW. Resting metabolic rate was measured by indirect calorimetry, and the thermic effect of a meal was estimated (10% of daily EE). Physical activity EE was obtained by subtracting the values for resting metabolic rate and thermic effect of a meal measured with DLW from those measured with the armband. Body composition was measured with dual-energy X-ray absorptiometry. Concordance between measures was evaluated by intraclass correlation, SEE, regression analysis, and Bland–Altman plots.

Results: Mean estimated daily EE measured with the armband was 117 kcal/d lower (2375 ± 366 kcal/d) than that measured with DLW (2492 ± 444 kcal/d; P < 0.01). Despite this group difference, individual comparisons between the armband and DLW were close, as evidenced by an intraclass correlation of 0.81 (P < 0.01).


KEY WORDS Energy expenditure, physical activity, doubly labeled water, portable armband

INTRODUCTION

Increasing daily energy expenditure (EE) plays an important role in the regulation of body weight and in the prevention or treatment of several lifestyle-related diseases, including mortality (1–7). Despite the critical importance of the appropriate level of daily EE and its central role in some theories of aging (8), in understanding the course of certain illnesses (9), and in the regulation of energy balance, its accurate measurement in free-living individuals remains difficult (5, 10, 11). There is a need to develop and validate instruments that could be useful in the monitoring of individual levels of total and physical activity EE. Furthermore, the self-monitoring of daily EE may increase awareness regarding the levels of EE needed to reduce health problems associated with physical inactivity (eg, obesity and type 2 diabetes) and serve as a useful element to promote lifestyle change (3, 4, 12).

Investigators have not consistently used a criterion method to validate instruments to measure daily EE. This is not a trivial point, because noncriterion methods are frequently compared against other noncriterion methods. This type of approach may suffer from a circularity in that the 2 methods compared (eg, 2 forms of self-report) may have correlated errors, which makes it difficult to arrive at a consensus regarding the accuracy of a given instrument. For example, questionnaires intended to evaluate physical activity EE have recently been shown to either over- or underestimate actual EE up to 60% (13). The development of the doubly labeled water (DLW) method has provided one valid and unobtrusive method for measuring free-living EE, from which the accuracy of field instruments can be determined (10). Unfortunately, its relatively high price, the need for mass spectrometry instrumentation, and the required technical expertise have limited its widespread application in research. Thus, the objective of this study was to examine the accuracy of a portable armband device and its associated software as a system to assess daily and physical activity EE in free-living adults in comparison with the DLW method.

SUBJECTS AND METHODS

Subjects

The physical characteristics of the men and women in the study sample are shown in Table 1. All subjects participated in a medical history and physical examination. To be eligible to participate, the subjects had to be ≥18 and ≤85 y of age, have no major illness or disease other than diabetes (type 1 or type 2), have a body mass index (BMI; in kg/m²) ≥18 and ≤35, be nonsmokers, and be low-to-moderate alcohol consumers (≤2drinks/d). We sought to include both persons in good health and a subset of diabetic persons. Fifty persons were recruited and
tested, but only 45 were included in the data analysis. To be included in the final data analysis, “on-body” time of the armband needed to be >95%. Five persons did not meet the criterion of >95% on-body time. The reasons for unacceptable time are not readily apparent, with the notable exception of one person, who had placed the armband on the arm in such a way that no contact with the skin was possible and thus no data were recorded. Of the 45 subjects included in data analysis, 13 were men (2 diabetic) and 32 were women (4 diabetic).

Overview of protocol

The study was approved by the University of Montreal Ethics Committee. All subjects were recruited on a voluntary basis and signed a consent form. A medical appointment was scheduled at the metabolic unit facility on day 0. DLW was administered on day 0, and urine samples were collected on days 0 and 1. The DLW measurement period was completed with urine samples collected on day 10. On day 1, the armband was strapped onto the right arm before the measurement of resting metabolic rate (RMR). EE was simultaneously measured by the DLW and armband methods for the 10-d period. RMR was measured in a controlled environment for 40 min with the ventilated-hood technique. After completion, the subjects were instructed on how to use the armband and how to complete a daily diary. The diary was used to record any problems with the armband and to document when the armband was removed (eg, for showering). The subjects were asked to make a third visit to the metabolic unit on day 1 when the armband was removed (eg, for showering). The sub-
jects were instructed to 1) fast and drink only water for 12 h before testing, 2) consume no alcohol, 3) restrain from physical activity for 24 h before testing, and 4) keep physical activity to a minimum on the morning of the test. Measurements were performed while subjects were lying in a supine position, without speaking or sleeping and with minimum movements. Measurements were performed over a 40-min period. The first 10 min were considered an acclimatization period, and data from the last 30 min were used for the analyses. The temperature of the room was maintained at an average of 22 °C. The calorimeter gas analyzers were calibrated before every measurement for pressure and gas concentrations. In our laboratory, the intraclass correlation coefficient (ICC; 2-factor random effect) for RMR determined by using a test-retest condition in 19 different subjects is 0.92 (P < 0.01). RMR was also calculated according to the World Health Organization (WHO) equations (15).

Thermic effect of a meal

The thermic effect of a meal (TEM) was estimated as 10% of daily EE and was calculated as daily EE × 0.10 (16). The calculation was specifically made for each daily EE measurement (by DLW or armband).

Body composition and anthropometric measurements

Body weight (kg) was measured to the nearest 20 g with an electronic scale (Balance Industrielles Montréal Inc, Montreal, Canada), and standing height was measured to the nearest 0.1 cm with a wall stadiometer (Perspective Enterprises, Portage, MI). Both measurements were performed following standard techniques while the subjects were shoeless. BMI was calculated as body weight (kg)/height2 (m). Fat-free mass and fat mass were measured by DXA with a LUNAR Prodigy system (software version 6.10.019; General Electric Lunar Corporation, Madison, WI). The DXA equipment was calibrated daily by using a known calibration standard. In test-retest analyses, the ICC (2-factor, random effect) in 18 different subjects was 0.99 (P < 0.01) for fat mass and 0.99 (P < 0.01) for fat-free mass.

Doubly labeled water

Daily EE was determined from DLW over a 10-d period. The DLW method uses the differential loss of the 2H and 18O isotopes of water to integrate carbon dioxide production over time in free-living subjects. After a dose of deuterated water is administered, the 2H is lost from the body water at an exponential rate. When 18O-water is administered, the 18O is also lost with body water turnover (as per the 2H isotope) and with each molecule of

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Variable</strong></td>
<td><strong>Value</strong></td>
</tr>
<tr>
<td>Age (y)</td>
<td>35.1 ± 14.0 (20.1–78.2)</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.69 ± 0.07 (1.58–1.88)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.2 ± 14.1 (52.0–107.1)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.9 ± 4.0 (17.9–34.3)</td>
</tr>
<tr>
<td>Fat-free mass (kg)</td>
<td>47.3 ± 8.6 (36.5–70.8)</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>18.4 ± 9.5 (2.8–43.3)</td>
</tr>
<tr>
<td>Daily EE by DLW (kcal/d)</td>
<td>2492 ± 444 (1606–3415)</td>
</tr>
<tr>
<td>Physical activity EE by DLW (kcal/d)</td>
<td>857 ± 326 (66–1514)</td>
</tr>
<tr>
<td>Resting metabolic rate (kcal/min)</td>
<td>0.96 ± 0.17 (0.72–1.49)</td>
</tr>
<tr>
<td>Physical activity level</td>
<td>1.6 ± 0.3 (1.03–2.4)</td>
</tr>
</tbody>
</table>

* All values are x ± SD; range in parentheses. EE, energy expenditure; DLW, doubly labeled water.
carbon dioxide produced because carbonic anhydrase in the body rapidly facilitates the equilibrium exchange of water and carbon dioxide/carbonic acid oxygen (17, 18). The difference between the rates of disappearance of $^2\text{H}$ and $^{18}\text{O}$ corresponds to the total carbon dioxide production over that period. These rates are determined from urine samples taken at the start and at the end of the measurement period. A fixed respiratory quotient of 0.88 was used to establish oxygen consumption and obtain a value for daily EE. The energy spent by each subject in physical activity per day can be estimated by subtracting other measured or estimated components (RMR and TEM) from daily EE (10).

The DLW experiments generated 5 urine samples per subject: a predose sample, 2 samples obtained after the $^2\text{H}_2^{18}\text{O}$ dose has initially equilibrated in the body (postdose samples 1 and 2), and 2 at the end of the collection period (postdose samples 3 and 4). Postdose samples 1 and 2 were collected on day 1, 16–24 h after the dose of DLW on day 0. There was a minimum of 30 min and a maximum of 4 h between postdose samples 1 and 2 as well as between postdose samples 3 and 4. All samples were measured in triplicate for $^{18}\text{O}$-water and in triplicate for $^2\text{H}$-water. An Isoprime Stable Isotope Ratio Mass Spectrometer connected to a Multiflow-Bio module for Isoprime and a Gilson 222XL Autosampler (GV Instruments, Manchester, United Kingdom) were used for daily EE measurements. Data processing was performed with MassLynx 3.6 software (Waters Corp, Milford, MA). Stability tests were performed each day before testing, which yielded an SD of 0.02% for deuterium and 0.004% for $^{18}\text{O}$. Known reference materials—Vienna-Standard Mean Ocean Water (V-SMOW), Greenland Ice Sheet Program (GISP), Standard Light Antarctic Precipitation (SLAP), and International Atomic Energy Agency standards (IAEA-304A and IAEA-304B)—were used for calibration and data normalization. Isotope ratio analysis results were reported as delta ($\delta$) relative to a reference gas. Daily EE was also determined with a prediction equation based on field variables (age, sex, and weight) (19).

**Statistics**

Statistical analyses were performed with SPSS software (version 13.0; SPSS Inc, Chicago, IL). Sample size calculations showed that 40 subjects (DLW versus the armband) would give 80% power at a 0.05 level test to reject the null hypothesis that the ICC is $\leq 0.60$ when the true underlying value is 0.80 and 2 measurements are performed in each subject. Thus, 50 subjects were enrolled in this study to plan for a 20% dropout rate due to subject noncompliance or other unanticipated problems. The ICCs are a class of statistics suitable for evaluating the extent of agreement between $\geq 2$ measures of the same construct (20). We used ICC (one-factor random effect). The ICC may be conceptualized as the ratio of between-subject variance to total variance. The closer the correlation is to 1.0, the greater the concordance between measures. To examine whether the differences between DLW and armband estimates are a function of true values, Bland and Altman analyses were used (21). Specifically, individual comparisons between DLW and the armband were completed by examining a plot of the differences in total and physical activity EE by DLW and the armband versus mean total and physical activity EE determined by both methods. From these data, limits of agreement between DLW and the armband were calculated, i.e., mean difference between DLW and the armband $\pm 2$ SD of the difference (daily EE: $\pm 454$ kcal/d; physical activity EE: $\pm 480$ kcal/d). A more stringent predefined value of $\pm 300$ kcal/d was set based on the fact that within-subject measures of daily EE (by DLW) can vary by 8% (or $\approx 200$ kcal/d) when accounting for analytic and biologic variation (22). Because we compared 2 methods (DLW versus armband) and not within-subject measures of DLW, we added an additional 100 kcal/d (200 + 100 kcal = 300 kcal/d) to estimate the number of outliers between methods.

A regression analysis was conducted between daily EE and physical activity EE between the armband and DLW. To evaluate the presence of a systematic bias, we also plotted the residual values against the reference method. Paired $t$ tests were performed to determine differences between the mean values obtained with the armband and DLW.

**RESULTS**

The physical characteristics and EE results from DLW for the 45 subjects ($n = 13$ men and 32 women) included in data analysis are shown in Table 1. The physical characteristics represent a relatively broad range of healthy and diabetic adult men and women.

### TABLE 2

<table>
<thead>
<tr>
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<th>Regression analysis ($r^2$)</th>
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<th>ICC ($r$)</th>
<th>ICC (95% CI)</th>
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<td>0.81$^2$</td>
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<td>DLW vs Vinken et al (19)</td>
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<td>0.14, 0.62</td>
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<td>0.37$^2$</td>
<td>0.09, 0.60</td>
<td>0.77</td>
</tr>
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<td>0.42$^2$</td>
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<td>0.64$^2$</td>
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</tr>
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<td>DLW vs RMR $\times 2.0$</td>
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<td>$\pm 395$</td>
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<td>0.78</td>
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$^1$ $n = 45$. ICC, intraclass correlation coefficient; DLW, doubly labeled water; RMR, resting metabolic rate.

$^2$ $P < 0.01$. 

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TABLE 3

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In triplicate for $^{18}\text{O}$-water and in triplicate for $^2\text{H}$-water. An Isoprime Stable Isotope Ratio Mass Spectrometer connected to a Multiflow-Bio module for Isoprime and a Gilson 222XL Autosampler (GV Instruments, Manchester, United Kingdom) were used for daily EE measurements. Data processing was performed with MassLynx 3.6 software (Waters Corp, Milford, MA). Stability tests were performed each day before testing, which yielded an SD of 0.02% for deuterium and 0.004% for $^{18}\text{O}$. Known reference materials—Vienna-Standard Mean Ocean Water (V-SMOW), Greenland Ice Sheet Program (GISP), Standard Light Antarctic Precipitation (SLAP), and International Atomic Energy Agency standards (IAEA-304A and IAEA-304B)—were used for calibration and data normalization. Isotope ratio analysis results were reported as delta ($\delta$) relative to a reference gas. Daily EE was also determined with a prediction equation based on field variables (age, sex, and weight) (19).

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### RESULTS

The physical characteristics and EE results from DLW for the 45 subjects ($n = 13$ men and 32 women) included in data analysis are shown in Table 1. The physical characteristics represent a relatively broad range of healthy and diabetic adult men and women.
women. Physical activity EE results showed that most subjects were lightly active, ie, had a mean physical activity level (PAL) of 1.6. The PAL was calculated as daily EE minus TEM divided by RMR. Because sex did not affect the results, all results are reported for the entire cohort.

The portable armband significantly underestimated (−117 kcal/d; \( P < 0.01 \)) daily EE (2375 ± 366 kcal/d) versus DLW (2492 ± 444 kcal/d) over a 10-d period (Table 2). We then compared DLW values with values obtained with widely used equations. The equation of Vinken et al (19) significantly overestimated daily EE (361 kcal/d; \( P < 0.01 \)), and the WHO equations (15) ranged from underestimating daily EE by 357 kcal/d to overestimating it by 558 kcal/d with the sedentary (1.4) and heavy (2.0) activity factors, respectively. The smallest mean difference was observed between DLW and the WHO equation multiplied by an activity factor of 1.6 (−52 kcal/d; \( P = 0.34 \)). This result can easily be explained by the actual average PAL of 1.6 of our population.

Although there was a significant group mean difference between the values obtained with the armband and DLW methods, individual values were relatively similar, as evidenced by the ICC of 0.81 (95% CI: 0.68, 0.89) in Table 3. This result indicates that 81% of the variance was explained by differences between individuals, whereas 19% of the variation was due to variation between methods. The individual agreement was not as high for the WHO value multiplied by an activity factor of 1.6 (ICC \( r = 0.64, P < 0.01 \)) or for the Vinken et al (19) equation (ICC \( r = 0.41, P < 0.01 \)). The magnitude of the potential discrepancy between the armband and DLW methods is shown by the SEE (±189 kcal/d). The scatterplot between the armband and DLW methods for total daily EE is presented in Figure 1. It must be noted that 2 measures can have a large difference in mean or variance and still have a perfect correlation. The regression analysis showed a moderate level of agreement between the DLW and armband measurements of daily EE (\( r^2 = 0.74, P < 0.01 \)). No apparent outliers were noted.
The Bland-Altman plot for total daily EE is shown in Figure 2. This plot examines comparisons between the armband and DLW by plotting differences in total daily EE between DLW and the armband versus mean daily EE determined between DLW and the armband. We noted that 80% of the values (n = 36 of 45) were within the predefined level of agreement between methods (±300 kcal/d) and 98% (n = 44 of 45) were within 2 SD (±454 kcal/d) of the difference between methods. Results of the regression analysis for residual values for TEE plotted against the values obtained from the DLW measurements are shown in Figure 3. The plot identifies an overestimation for the lower EE results and an underestimation of the higher EE values (r² = 0.33, P < 0.01). A significant mean difference was observed between physical activity EE measured with the armband versus DLW (Table 4; mean difference of −225 kcal/d; P < 0.01; n = 41). The ICC between these 2 measures was r = 0.46 (95% CI: 0.19, 0.67; P < 0.01), which suggests that 46% of the variance was explained by differences between individuals and 54% of the variation was due to variation between methods. The SEE was ±179 kcal/d. Similar comparisons were made for the physical activity EE calculated with the equation of Vinken et al (19) and the WHO equations multiplied by an activity factor (daily EE from equations minus indirect calorimetry measurements for RMR and calculated TEM). Individual agreement was better with the armband measurements (ICC r = 0.46, P < 0.01) than with any other method. The smallest mean difference was observed for the WHO equations multiplied by 1.6 (−40 kcal/d; P = 0.44). The regression analysis between the armband and DLW methods for physical activity EE was significant (r² = 0.49, P < 0.01; n = 41), as shown in Figure 4. The concordance between the armband and DLW methods for measuring physical activity EE, with the use of Bland-Altman plots, is shown in Figure 5. We noted that 83% (n = 34 of 41) of the subjects were within 2 SD of physical activity EE. The armband was either placed improperly or malfunctioned during the measurement of RMR for 4 of the 45 subjects included in the data analysis. Thus, these 4 subjects did not have values available for this analysis and were excluded.

Although it was not the major purpose of this study, we reported a concordance between measures of indirect calorimetry and the armband measurement (data not shown in tabular form). The armband provided higher values for RMR (1.05 ± 0.17 kcal/min) than did indirect calorimetry (0.96 ± 0.17 kcal/min; P < 0.05), which suggests a possible overestimation with the armband. The ICC between these 2 measures was 0.77, which suggested that 77% of the variance was explained by differences between individuals, whereas 23% of the variation was due to variation between methods. The regression analysis between the armband and indirect calorimetry was r² = 0.77 (SEE = ±0.08 kcal/min; P < 0.01). Similar results were obtained when the WHO equations were used to predict RMR (r² = 0.79, SEE = ±0.08 kcal/min; P < 0.01).

**DISCUSSION**

We evaluated the accuracy of the portable armband compared with that of the DLW method for measuring both total daily and
physical activity EE in free-living adults. Our findings suggest reasonable concordance between the methods on the basis of ICCs and the Bland-Altman plots.

Total energy expenditure

Despite an average significant underestimation of daily EE by the armband (−117 kcal/d) compared with the DLW method, the methods provided relatively similar results. This statement is based on the magnitude of the ICC, which showed that 81% of the variance in daily EE was explained by interindividual differences (Table 3) and a high correlation coefficient between methods (Figure 1). Daily EE values for 9 of the 45 subjects were outside the hypothesized ±300 kcal/d range. We compared the physical characteristics of these 9 subjects with those of the 36 subjects who had values within the acceptable range of prediction and found no differences in any of the physical characteristic between these groups. Thus, we cannot explain why these 9 subjects were “outliers,” although one may suspect that sources of discrepancies include the armband’s ability to accurately record different types of physical activity. However, the residual values (Figure 3) showed that the armband yielded an overestimation of daily EE for the subjects with low EE values and an underestimation of daily EE for the subjects with high EE values. These findings suggest that the device is more accurate for usual daily EE than for extreme levels (eg, athletes).

Nonetheless, despite a tendency to underestimate mean values, the armband provided better results than did common field methods for measuring daily EE (15, 19). Thus, the device may be a useful clinical instrument to estimate or monitor daily EE. For example, if threshold levels of daily EE could be estimated and prescribed to individuals on the basis of a person’s age or diagnosis (eg, obesity or type 2 diabetes), the armband could provide useful feedback for a person seeking to increase and maintain a higher threshold level of daily EE. Accurate

FIGURE 4. Regression analysis between the armband and doubly labeled water methods for measuring physical activity energy expenditure (n = 41; P < 0.01).

FIGURE 5. Bland-Altman plot between the armband and doubly labeled water methods for measuring physical activity energy expenditure (n = 41). The unbroken horizontal lines represent the limits of agreement corresponding to ±2 SD. The broken horizontal lines represent the ±300-kcal limits of agreement.
self-monitoring in the free-living environment may provide helpful feedback that increases self-awareness—an important element for positive decision making and positive lifestyle changes (3, 4, 12). These types of studies, however, need to be performed in larger and more diverse sample populations (eg, children, older adults, obese persons, and athletes) to determine whether the armband is accurate in various population subgroups and assists in increasing subject compliance. In addition, we cannot provide information on whether the armband accurately measures EE over a single day. Although this instrument does provide this type of information, DLW does not. A limitation of the DLW approach is that it provides an integrated assessment of daily EE and not a day-to-day assessment of EE. In this respect, the armband might give timely additional information to assist the subject in regulating energy balance.

Physical activity energy expenditure

The measurement of physical activity EE with the armband was somewhat less precise than was the measurement of total daily EE. On the basis of the ICC, we noted that 46% (compared with 81% for daily EE) of the variation was explained by interindividual differences and 54% (compared with 19% for daily EE) of the variation was explained by interdaily EE. On the basis of the ICC, we noted that 46% (compared with 81% for daily EE) of the variation was explained by interindividual differences and 54% (compared with 19% for daily EE) of the variation was explained by interdaily EE. However, a similar level of concordance was obtained when WHO equations were used to predict RMR (ICC $r = 0.75, P < 0.01$). These similar results were probably due to the fact that the variables used (sex, weight, and age) were similar between both the WHO equations and the armband.

It should be noted that no important safety issue was reported regarding the use of the armband. Four subjects mentioned only minor discomfort during sleep or small skin irritations toward the end of the 10-d wearing period. No lesions were apparent on medical inspection.

In conclusion, the results of the present study support a reasonable level of concordance between the portable armband and DLW methods, especially for measuring total daily EE and, to a lesser degree, physical activity EE. This portable device might be useful in helping individuals to increase their level of daily EE to regulate energy balance and energy needs and to offset chronic diseases associated with physical inactivity (1, 23).

We thank Jean-Marc Lavoie from the Department on Kinesiology of the University of Montreal for his contribution to the analysis and interpretation of the results.

MS-O collected and analyzed the data and wrote the draft of the manuscript. DM analyzed the DLW data and revised the manuscript. DBA conducted the statistical analysis and revised the manuscript. RR-L supervised the study, wrote the draft of and revised the manuscript. RR-L and DBA received honorarium from the sponsor Roche Diagnostics. MS-O was involved with a private company that used the armband founded after the completion of the study. DM declared no conflict of interest.

TABLE 5
Level of agreement between measures of physical activity energy expenditure

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Regression analysis ($r^2$)</th>
<th>SEE</th>
<th>ICC</th>
<th>ICC (95% CI)</th>
<th>Cronbach’s $\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLW vs armband</td>
<td>0.49$^2$</td>
<td>±179</td>
<td>0.46$^2$</td>
<td>0.19–0.67</td>
<td>0.80</td>
</tr>
<tr>
<td>DLW vs Vinken et al (19)</td>
<td>0.15$^2$</td>
<td>±186</td>
<td>−0.003</td>
<td>−0.29–0.29</td>
<td>0.52</td>
</tr>
<tr>
<td>DLW vs RMR × 1.4</td>
<td>0.11$^3$</td>
<td>±148</td>
<td>−0.10</td>
<td>−0.38–0.20</td>
<td>0.40</td>
</tr>
<tr>
<td>DLW vs RMR × 1.6</td>
<td>0.09$^3$</td>
<td>±183</td>
<td>0.26$^3$</td>
<td>−0.03–0.51</td>
<td>0.41</td>
</tr>
<tr>
<td>DLW vs RMR × 2.0</td>
<td>0.06</td>
<td>±263</td>
<td>−0.27</td>
<td>−0.52–0.03</td>
<td>0.40</td>
</tr>
</tbody>
</table>

$^1 n = 41$. ICC, intraclass correlation coefficient; DLW, doubly labeled water; RMR, resting metabolic rate.

$^2 P < 0.01$.

$^3 P < 0.05$.

Resting components of total daily energy expenditure

The ICC was $r = 0.77 (P < 0.01)$ for RMR, which suggests that $>77\%$ of the individual differences were due to interindividual differences. However, a similar level of concordance was obtained when WHO equations were used to predict RMR (ICC $r = 0.75, P < 0.01$). These similar results were probably due to the fact that the variables used (sex, weight, and age) were similar between both the WHO equations and the armband.

REFERENCES


Calcium balance in 1–4-y-old children

Mary Frances Lynch, Ian J Griffin, Keli M Hawthorne, Zhensheng Chen, Maria Hamzo, and Steven A Abrams

ABSTRACT

Background: Few calcium balance data are available from young children on which to base dietary recommendations.

Objective: The objective of the study was to evaluate the relation between calcium intake and balance in healthy children aged 1–4 y consuming typical American diets.

Design: Subjects were assigned to a diet with nutrient intakes similar to those of their usual diet. Calcium absorption was assessed by using a dual-tracer stable-isotope technique. Endogenous fecal excretion was measured in a subset of children, and net calcium balance was calculated.

Results: Mean calcium intake was 551 mg/d (range: 124–983 mg/d), and mean (±SEM) calcium retention was 161 ± 17 mg/d. Both linear and nonlinear modeling of balance data showed that a calcium intake of ≈470 mg/d led to calcium retention of 140 mg/d, which is the amount that meets expected bone growth needs in children of this age. No evidence was found that calcium intakes of 800 to 900 mg/d reached the threshold intake beyond which no additional increase in calcium retention would occur.

Conclusions: Bone growth needs in 1–4-y-old children following American diets are met by a daily calcium intake of ≈470 mg/d, which suggests that the current Adequate Intake of 500 mg/d is close to the actual Estimated Average Requirement. The benefits and risks of higher calcium intakes consistent with threshold values should be evaluated in a controlled trial before those intakes could be used as a basis for dietary recommendations. Am J Clin Nutr 2007;85:750–4.

KEY WORDS Calcium absorption, stable isotopes, bioavailability, nutrient requirements

INTRODUCTION

In 1997, new dietary guidelines for minerals (including calcium) were released by the Institute of Medicine. At that time, because a perception existed of limited available data on which to base an Estimated Average Requirement (EAR) and a Recommended Dietary Allowance (RDA) for calcium, intake recommendations were limited to the use of an Adequate Intake (AI) and a Tolerable Upper Limit (UL) (1). Since 1997, considerable data on calcium requirements have been reported for most population groups, and it is reasonable to believe that an EAR could be established for adults and adolescents (2, 3). This possibility has clear importance because the EAR is an important aspect of food labeling and dietary planning guidelines (4, 5).

One group for which minimal data have been available is young children, especially those who are <4 y old (1, 6). It is well recognized that dietary patterns are different in this age group than in infants or older children (1, 2, 4, 7), and because of this difference, special food-labeling guidelines are in place for these age groups (4). However, no data are available on which to rationally base a calcium EAR for this age group. Essentially, all recommendations have adapted data obtained in other age groups (1) or have combined the <4-y-old group with children aged 4–8 y to estimate calcium requirements for prepubertal children (8, 9). The minimal available data on calcium balance in young children were generated many years ago; those data may have substantial systematic errors and do not reflect current typical diets (6, 10).

The principal reason for the absence of data in this age group is the impracticality of prolonged dietary regulation and complete urine and fecal collections that are required for traditional balance studies especially in active children who are often not toilet-trained. Studies in this age group are now more feasible with stable-isotope methods in which calcium absorption can be directly assessed with a short-term urine collection (11).

Further complicating the understanding of dietary calcium requirements for young children is the fact that the usual daily intake in the United States (median: 766 mg/d) is much higher than the AI of 500 mg/d (1). Therefore, in the current study, our goals were to evaluate the relations among calcium intake, absorption, and retention in healthy young children across a range of dietary calcium reflecting both the AI and typical intakes and to relate these data to bone growth needs so as to develop a candidate EAR.

SUBJECTS AND METHODS

Subjects

Healthy children aged 12–48 mo residing in the greater Houston area were recruited through public advertising. Subjects were selected to reflect the approximate racial and ethnic distribution of

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the greater Houston area. No subjects aged 12–14 mo were enrolled, and thus the final age range for the subjects was 15–48 mo.

Written informed consent was obtained from each subject’s parents for all studies. The Institutional Review Board of Baylor College of Medicine and Affiliated Hospitals approved the protocol.

Methods

Screening visit

Children were eligible for enrollment if they were healthy, were not taking any medications except multivitamins, were born at term (≥37 wk gestation), and had a birth weight ≥2500 g. Children were excluded from participating if they had chronic health problems, were below the 3rd or above the 97th percentile of weight or height-for-age, or were below the 5th or above the 95th percentile of weight-for-height. Those subjects taking multivitamins were required to discontinue their use 2 wk before their participation in the mineral absorption study. Families were offered the option of participating in a 2-d calcium absorption study or a 5-d study in which fecal samples would also be collected and endogenous excretion would be measured.

The research dietitian met with each subject’s parents and obtained a 24-h diet history to evaluate the usual daily micronutrient and calorie intake. To reflect the marketplace, dietary intake data were analyzed with the use of NUTRITION DATA SYSTEM FOR RESEARCH software (versions 5.0 and 2005; Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN). Final calculations were completed with the use of version 2005.

A plan was developed for the diet that each child would consume at home for the 7 d before the inpatient mineral absorption study. This step was taken to ensure that children did not alter their eating habits immediately before the mineral absorption study. All foods and beverages to be consumed during these 7 d were provided by the research center and were weighed before delivery to the family for the period before the mineral absorption study (the prestudy period). Parents were instructed to return all uneaten food and beverage items from the first 3 d of the week-long prestudy period so that the unconsumed items could be weighed.

Isotope preparation and mineral absorption study

We purchased $^{42}$Ca (94% enrichment) and $^{46}$Ca (6% enrichment) from Trace Sciences (Toronto, Canada), and they were prepared for human use as the chloride salt by the Investigational Pharmacy Service of Texas Children’s Hospital (TCH), Houston, TX. All isotopes were tested for sterility and pyrogenicity before use.

At the end of the home pack-out period, patients were admitted to the General Clinical Research Center at TCH for the calcium absorption study. On the morning of the inpatient study, a heparin-lock intravenous catheter was placed with the use of topical 4% lidocaine cream (L-M-X-4; Ferndale Laboratories, Ferndale, MI) as an analgesic. Subsequently, 15 μg $^{46}$Ca was given intravenously over 1 min. The subgroup of subjects in whom endogenous fecal calcium was being measured received a larger dose, 40 μg $^{46}$Ca, which was given intravenously.

Subjects were then given breakfast that included 30 mL calcium-fortified apple juice (Minute Maid; Coca-Cola Company, Atlanta, GA). The juice contained 15 mg Ca to which a further 2 mg $^{42}$Ca had been added. After the subject consumed the isotope-containing juice, the subject consumed another 30 mL calcium-fortified apple juice without isotope from the same cup as a rinse to ensure that none of the isotope was left in the cup. This process was repeated at lunch with the use of the same amount of isotopes as at breakfast.

Each breakfast provided approximately one-third of the daily mineral intake of the subject. The remaining intake was divided between lunch, dinner, and 2 small snacks. Menus for the inpatient study visit were based on each subject’s usual mineral intake that he or she had received at home for the previous 7 d. All foods and beverages provided during the inpatient visits were weighed before the visit, and the unconsumed foods and beverages were weighed after the visit to measure intake. Dietary intakes used in the results section were based on these intakes.

Subjects remained in the inpatient unit for 48 h, and their urine was collected in 24-h pools for the duration of their hospitalization. If the subject was not well toilet-trained, urine bags were used for the sample collection. The subset of subjects in whom endogenous fecal excretion was measured remained in the inpatient unit for 120 h, during which time their urine and stools were collected in 24-h pools.

Calculation of mineral absorption

Urine samples were prepared for mass spectrometric analysis by using an oxalate precipitation technique as previously described (11). Samples were analyzed for isotopic enrichment with the use of a magnetic sector thermal ionization mass spectrometer (Finnigan MAT 261; Finnigan, Bremen, Germany). Each sample was analyzed for the ratio of $^{42}$Ca to $^{43}$Ca and $^{46}$Ca to $^{44}$Ca with correction for fractionation to the reference $^{43}$Ca to $^{44}$Ca. The accuracy and precision of this technique for natural abundance samples compared with those of standard data are ≥0.15%, depending on the ratio being measured.

Calcium absorption was calculated as the relative recovery in the urine of the oral isotope divided by the recovery of the intravenous isotope during the 24 h after isotope administration (from time of the first oral dose until 24 h after the last oral dose). The endogenous fecal excretion of calcium was calculated as the ratio of urinary to fecal recovery of the intravenously administered isotope by using the equations described previously (12, 13). Mineral balance was calculated as the difference between total dietary absorption (the product of intake and fractional absorption) and the sum of urinary and endogenous fecal excretion.

Sample size and statistical analysis

Data were analyzed by using SPSS for WINDOWS software (version 13; SPSS Inc, Chicago, IL. Power calculations were carried out by using DSTPLAN (version 4.2; MD Anderson Cancer Center, Houston, TX). All results are shown as means ± SEMs.

Previous studies suggested a correlation coefficient ($r$) of 0.5 between calcium intake and net calcium balance in this age range (6). A sample size of 25 has an 80% power of detecting this degree of correlation at $P < 0.05$.

RESULTS

Subject demographics

Calcium absorption was measured in 28 subjects (14 boys and 14 girls). The ethnic distribution of the study population was 46%
white, 29% Hispanic, 18% African American, and 7% multiethnic. Ethnicity and sex were initially considered as a covariate in each analysis but did not significantly affect the relations between calcium intake and absorption or excretion and were omitted from further analyses. The baseline characteristics of the study group are shown in Table 1. Eight subjects agreed to and completed the 5-d studies in which endogenous fecal calcium excretion was measured. The baseline characteristics of this subgroup did not differ significantly from those of the group as a whole (mean age: 26±3 mo; weight: 12.5±0.8 kg; height: 87.0±2.8 cm; calcium intake: 563±70 mg/d). No significant correlation was found between calcium intake and body weight, height, or age for the study subjects (P>0.10 for each).

### Relation between calcium absorption and retention

Average calcium intake in study subjects during the mineral absorption study was 551±41 mg/d (median: 513 mg/d). Calcium intakes during the prestudy home dietary period did not differ significantly from those during the study and are not shown. Calcium fractional absorption was significantly and negatively correlated to intake (r = -0.50, P = 0.006) (Figure 1). The use of nonlinear modeling did not increase the correlation between intake and retention. Mean calcium absorption was 45.6±2.5% of intake.

To determine calcium retention, measurement of endogenous fecal excretion in each subject was necessary. We used the mean value obtained from the 8 subjects in whom endogenous fecal excretion was measured (ie, 3.5 mg·kg⁻¹·d⁻¹) for the other 20 subjects, in whom it was not measured. A fixed value was used rather than one based on calcium intake, because no significant

### Relation between calcium intake and retention

We evaluated the relation between calcium intake and calcium retention by using both linear and nonlinear models. With the use of linear regression analysis, the relation between intake and retention was fitted by the following equation:

\[
\text{Calcium retention} = 0.25 \times (\text{calcium intake}) + 23.6
\]

where \( r = 0.60 \). An improved fit (\( r = 0.75 \)) was obtained by using an S-curve relation (Figure 2). No close relation was found between calcium intake and fractional retention (\( r = 0.05 \)).

### Relations between calcium retention, requirements for growth, and the threshold value

Available data, including weight-based estimates published in the 1950s (14) and dual-energy X-ray absorptiometry (DXA) bone mineral data, showed that calcium accretion to the whole skeleton averages 100–120 mg/d from 1–4 y of age (15, 16). Because calcium balance as performed with isotopes or by using traditional mass-balance techniques does not include dermal (sweat) losses, the dermal loss value must be estimated and added to the skeletal accretion value to determine the requirement for daily calcium retention (1, 8). Estimates of dermal losses of 20 to 40 mg are generally used for prepubertal children (1, 8). Therefore, a mean value of 140 mg/d can be estimated as the required average calcium balance (110 mg/d bone calcium accretion and 30 mg/d dermal loss).
TABLE 2
Calcium intakes needed to achieve various calcium retention (balance) values

<table>
<thead>
<tr>
<th>mg/d</th>
<th>Retention of 100 mg/d</th>
<th>Retention of 140 mg/d</th>
<th>Retention of 180 mg/d</th>
<th>Retention of 200 mg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear (this study)</td>
<td>305</td>
<td>465</td>
<td>625</td>
<td>705</td>
</tr>
<tr>
<td>S curve (this study)</td>
<td>332</td>
<td>472</td>
<td>691</td>
<td>857</td>
</tr>
<tr>
<td>Matkovic and Heaney (6)</td>
<td>603</td>
<td>771</td>
<td>939</td>
<td>1023</td>
</tr>
</tbody>
</table>

1 Values are derived in which calcium intake values (x, mg/d) are shown to achieve various levels of calcium retention (y, mg/d). The equation for the linear relation was y = -0.00031 × x + 0.626 (r² = 0.36), and that for the S curve was y = Exponent [5.732 + (-373/x)] (r² = 0.55); P < 0.001 for each.

The calcium intake, calculated from our data and the earlier data (6), needed to achieve a range of calcium retention values is shown in Table 2. Both models of our data show that an intake of ≈470 mg/d is required to achieve the 140 mg/d calcium retention needed to meet bone accretion needs and to offset dermal losses.

Another measurement of interest is the calcium intake at which calcium retention reaches or approaches a maximum (1, 2, 8). This value is referred to as a threshold value. Too few subjects in our study had intakes high enough to allow us to estimate this value. Our data (Table 2 and Figure 2) suggest that the threshold intake is ≥850–900 mg/d.

DISCUSSION

We have shown that calcium absorption in 1–4-y-old children is inversely related to usual dietary intake. The amount of retained calcium needed for bone growth according to average rates of bone mineral accretion is achieved at calcium intakes of ≈470 mg/d. This intake is much lower than the amount needed by adolescents for bone growth or the amount needed by adults to prevent bone loss (1, 14, 17). Compared with adults, young children have significantly higher calcium absorption efficiency and significantly lower rates of total calcium excretion (1, 14, 17).

The lack of data in this age group has led to a wide range of estimates for the recommended calcium intake. In the United States, the reference calcium intake is 500 mg/d, which is an AI; and at the time that the AI was set, it was believed that data were not available from which to establish EAR and RDA values (1). However, the AI concept is very limiting for setting dietary policy, and EAR values are available for many other minerals, such as magnesium, for which far fewer balance data are available than for calcium. It is important that an EAR be established for use in processes including revision of food labels and meal planning (4, 5). Establishment of an EAR is likely possible at this time in older age groups because of the large amount of new data generated since the Dietary Reference Intakes process of the mid-1990s, but no similar data have been generated for young children (2, 3, 17).

In general, despite the disparity of techniques and populations studied, estimates of calcium accretion by the skeleton in early childhood have a reasonable consistency over the past 50 y. Mean calcium accretion averages ≈80 mg/d during the first year of life, increases slightly to ≈100 mg/d during the second year of life (15), and increases to ≈120 mg/d during the preschool and early school years (1, 8, 14–16).

Calcium’s absorptive efficiency averaged 46% for a population mean intake of 551 mg/d. This is ≈1.5 SD above the value for estrogen-replete premenopausal women (ie, ≈30 ± 10%) at the same calcium intake (18, 19). Thus, although calcium’s absorptive efficiency has been shown to be greater in adolescents than in adults, it appears that, at low calcium intakes, the same is true for infants and young children (20, 21).

Our data indicate that the current AI of 500 mg/d is very close to the actual “average requirement” needed to meet average bone growth requirements. Reasonable rounding of our value would lead to a candidate EAR of 500 mg/d for children 1–4 y of age. Assuming that an additional 30% calcium retention (a total retention of ≈180 mg/d) would meet the needs of 97.5% of the population, the best fit of our data would suggest a candidate RDA of ≈700 mg/d.

Around the world, wide ranges of dietary recommendations exist for calcium in young children. Recommendations generally are expressed as a value whose definition is similar to the RDA—ie, an intake that meets the needs of nearly all members of the population. In the United Kingdom, this value is 350 mg/d, whereas, for the European Union, it is 400 mg/d. A much higher value of 800 mg/d is recommended in Ireland. The calcium intake recommended by the FAO/WHO is 500 mg/d (9, 22). In the United Kingdom, as in the United States, mean calcium intakes are far above the recommended amount, which makes it difficult to assess the consequences of low intakes (22). Ultimately, however, this range of recommendations, more than fundamental variations in diets or genetics, reflects the lack of usable data in this age group.

Another possible approach toward establishing dietary intake recommendations for calcium in children is to identify the threshold intake value—rather than using the average bone growth requirement—as the goal. Various methods may be used to estimate an approximate threshold or determine an intake at which near-threshold retention may reliably be achieved (1, 2, 6, 19). We cannot apply such approaches to our dataset, which is limited by both the small number of studies and the range of intakes. Our data, however, do not provide any evidence for a threshold at intakes of ≈850–900 mg/d.

With the use of a large database of studies mostly performed in the early part of the 20th century, a threshold of 1390 mg/d in children aged 2–8 y was reported (6). The threshold balance of 246 mg/d achieved at this intake was much higher than the usual rate of bone mineral calcium accretion by the skeleton. Although earlier balances likely had falsely elevated retention values for most intakes, the threshold calcium intake may not be affected by systematic errors related to short-term intake transients across the range of intakes (6). Therefore, in contrast to adolescents, in whom there is reasonable convergence between the calcium intake that meets growth needs and the threshold intake, these 2 values in infants (6, 21) and young children (the current study) are markedly different. It is important to note that no data have suggested any short or long-term benefit from high rates of calcium retention in children of this age. Thus, until further data are available, the goal of meeting average bone growth requirements is appropriate (1).

Ultimately, identifying an “optimal” intake in young children, as in infants, can be accomplished only by long-term studies...
evaluating the benefits and potential risks of intakes that are above the usual growth needs (23). Such data are extremely difficult to generate, however, and doing so requires studies that begin in the first few years of life and provide follow-up to at least early adolescence. Therefore, revision of the dietary requirements to establish an EAR for calcium cannot await data that may not be available for many decades.

A limitation of our data is the lack of endogenous fecal excretion data in all subjects. However, results in the subjects in which it was obtained are similar to those in previous reports in children (11, 12). It is extremely unlikely that substantial variation occurs in endogenous fecal excretion in young children, and the use of the mean data for nonmeasured subjects should lead to a small potential error in calculated balance. Other limitations are the use of databases to measure the calcium content of the ingested food and the lack of assessment of vitamin D status in these children. We did not find a relation between vitamin D status and calcium absorption in vitamin D–nondeficient older children, and it is unlikely that any of these children were vitamin D deficient (24).

Because of the global existence of both vitamin D and calcium deficiency rickets in the age group we studied, it is important also to consider the effects of very low calcium intakes. Our data indicate that calcium intakes of ~300 mg/d are consistent with retention of ~80–100 mg/d, an amount that is likely to represent an approximate minimum for bone growth (after dermal losses) in young children. In areas where large amounts of inhibitors of calcium absorption are present in the diet, a higher minimal intake may be needed. However, in some of those areas, urinary calcium excretion is very low, and, therefore, in the presence of adequate vitamin D, intakes of 300 mg/d likely are adequate (24, 25). Further data are needed in these settings to assess calcium requirements in populations with habitual intakes of high amounts of inhibitors of calcium absorption, and our data cannot be reliably used as a basis for dietary guidelines there.

In summary, we have shown that calcium intake and absorption efficiency are inversely related in young children and that a calcium intake of ~470 mg/d meets the usual bone growth needs of such children. This value, or the rounded value of 500 mg/d, may be considered as a candidate EAR value for 1–4-y-old children.

The authors thank the nursing staff of the General Clinical Research Center of Texas Children’s Hospital for caring for the study subjects and the investigational pharmacy of Texas Children’s Hospital for preparation of the isotopes.

SAA was responsible for the overall conduct of the study; IJG and MFL for daily study supervision; KMH supervised all dietary aspects of the study; MH was responsible for the laboratory analysis of total minerals and assisted in mass spectrometric analyses; ZC was responsible for the protocol design and the laboratory analysis of stable isotope studies; and all authors were involved in the preparation of the manuscript. None of the authors had any personal or financial conflict of interest.

REFERENCES


ABSTRACT

Background: Inadequate vitamin D status is common in many populations around the world.

Objective: The aim was to evaluate potentially modifiable determinants of vitamin D status in an older population.

Design: This was a cross-sectional study from a population-based cohort including 538 white Dutch men and women aged 60–87 y. Vitamin D status was assessed by plasma 25-hydroxyvitamin D [25(OH)D] concentrations.

Results: In the winter period, 51% of the subjects had 25(OH)D concentrations <50.0 nmol/L. Greater body fatness and less time spent on outdoor physical activity were associated with worse vitamin D status. Regular use of vitamin D–fortified margarine products [odds ratio (OR) in a comparison of intake of ≥20 g/d with none: 0.41; 95% CI: 0.20, 0.86; P for trend < 0.001], fatty fish (OR for servings of ≥2/mo versus none: 0.41; 95% CI: 0.16, 1.04; P for trend = 0.01), and vitamin D–containing supplements (OR for ≥1/d versus none: 0.33; 95% CI: 0.17, 0.63; P for trend < 0.001) were inversely associated with vitamin D inadequacy [25(OH)D <50.0 nmol/L]. We estimated that combined use of margarine products (20 g/d), fatty fish (100 g/wk), and vitamin D supplements (≥1/d) was associated with a 16.8 nmol/L higher 25(OH)D concentration than was the use of none of these. However, none of the participants reached these intakes for all 3 factors.

Conclusion: Because few foods are vitamin D–fortified and the amounts of vitamin D in supplements are low, it is difficult to achieve adequate vitamin D status through increasing intakes in the Netherlands and in countries with similar policies. Am J Clin Nutr 2007;85:755–61.

KEY WORDS Vitamin D, food fortification, supplement use, body fatness, population-based study

INTRODUCTION

The importance of vitamin D for bone health and muscle function has long been acknowledged, and accumulating evidence suggests that adequate vitamin D status may contribute to the prevention of autoimmune diseases, hypertension, and various types of cancer (1). Inadequate vitamin D status can be subdivided into “vitamin D insufficiency,” characterized by elevated serum parathyroid concentrations and a mild increase in bone turnover, and “vitamin D deficiency,” characterized by high bone turnover and possible bone mineralization defects (2). Vitamin D insufficiency is highly prevalent in many populations around the world (3), and vitamin D deficiency is common in institutionalized elderly (2) and Europeans of non-Western origin (4, 5).

Vitamin D is derived from dietary sources or from endogenous production in the skin under the influence of sunlight exposure (6). Possible measures to improve vitamin D status can target consumption of vitamin D–rich foods, fortification of foods, use of dietary supplements, or habits related to sun exposure. Vitamin D fortification of foods varies widely across the world: in the Netherlands, margarine products are the only foods that are vitamin D fortified; in the US and Canada, milk is generally vitamin D fortified; and in many other countries, foods are rarely vitamin D fortified (7). Currently, there is no consensus about the optimal strategy to improve the vitamin D status of populations, and information on the importance of various potentially modifiable determinants of vitamin D status may help to identify promising interventions. Few studies have examined the consumption of various foods in relation to vitamin D status (8, 9), and the effect of vitamin D fortification can be well studied in the Netherlands, because margarine products, but not other foods, are consistently fortified with vitamin D. We therefore examined determinants of vitamin D status in a population-based study of older men and women in the Netherlands, with a focus on dietary factors.

SUBJECTS AND METHODS

Study population

The present study was based on cross-sectional data from white men and women aged 60–87 y who participated in the 2000–2001 Hoorn Study follow-up examination (10). The
Hoorn Study is a population-based cohort study of glucose metabolism that started in 1989. The baseline examination was conducted in a random sample obtained from the municipal registry of the town of Hoorn, The Netherlands. From January 2000 through July 2001, a follow-up examination was conducted in surviving participants who had given permission to be recontacted. We invited all participants who had type 2 diabetes mellitus, and a random sample of those with impaired and normal glucose metabolism, at the previous follow-up examination in 1996–1998. Of those invited, 648 (60%) participated. The reasons that were given for nonparticipation were lack of interest (30%), morbidity (23%), old age (7%), unwillingness to travel (6%), participation considered too time-consuming (6%), and miscellaneous reasons (15%); 13% did not provide reasons for nonparticipation. After exclusion of persons with clinical type 2 diabetes ($n = 67$) who may have changed their lifestyle or reporting thereof as a result of the physician diagnosis and persons with missing data for any of the studied variables ($n = 43$), 538 persons remained for the current analysis. All participants provided written informed consent, and the study was approved by the Ethics Committee of VU University Medical Center Amsterdam.

**Measurements**

Vitamin D status was assessed by measuring 25-hydroxyvitamin D [25(OH)D] concentrations in fasting EDTA-plasma by using a competitive binding protein assay (DiasSorin, Stillwater, MN). This concentration is considered the most accurate measure of vitamin D status (11). The assay measures both 25-hydroxyvitamin D$_2$ and 25-hydroxyvitamin D$_3$, but will primarily represent 25-hydroxyvitamin D$_3$ concentrations in the current study, because vitamin D in the Dutch diet is mostly vitamin D$_3$. Laboratory measurements were conducted at the Endocrinology Laboratory, Department of Clinical Chemistry, VU University Medical Center Amsterdam. The interassay CV was 10–15%, with a slightly lower CV at higher concentrations. We performed a whole-body dual-energy X-ray absorptiometry scan using fan beam technology (QDR-2000, software version 7.20D; Hologic, Brussels, Belgium) to assess the percentage of body fat (10). Cigarette smoking habits and education level were assessed by self-administered questionnaires. High education level was defined as vocational colleges and university, medium level as secondary education, and low level as elementary school, lower vocational training, or less. Usual food consumption and supplement use and physical activity, including commuting activities, leisure time activities, and occupational activities, were assessed by using validated questionnaires (12, 13). Vitamin D–containing supplements included vitamin A and D supplements, calcium and vitamin D supplements, and multivitamins, which were the most commonly used vitamin D–containing supplements in the Netherlands. Dairy was considered to be high-fat if it contained >2% of weight as fat. “Fatty fish” included, for example, eel, mackerel, and herring, whereas “lean fish” included cod, plaice, mussels, and shrimp. Time spent on outdoor physical activity was estimated by adding the time spent on walking, cycling, and gardening.

**Statistical analysis**

Vitamin D status was categorized by using previously defined cutoffs (2). Although higher cutoffs have been proposed (1), we defined “vitamin D inadequacy” as 25(OH)D concentrations <50 nmol/L for the purpose of the current analysis. Statistical analyses were conducted by using SAS software version 8.2 (SAS Institute, Cary, NC). Differences by sex and season were evaluated by using Student’s $t$ test for continuous variables, the Wilcoxon’s rank-sum test for continuous variables that were not normally distributed, and a chi-square test for categorical variables. We performed linear regression analysis to derive regression coefficients for analyses with 25(OH)D concentrations as the dependent variable and logistic regression analysis to derive odds ratios for analyses with vitamin D inadequacy as the dependent variable. To address potential confounding by other determinants of vitamin D status, we used a multivariable model that included age (y), sex (man or woman), body fat (percentage), cigarette smoking (current, past, or never), education level (high, medium, or low), use of vitamin D–containing supplements (none, <1/d, or ≥1/d), outdoor activities (h/d), season (December to February, March to May, June to August, or September to November), total energy intake (kJ/d), and consumption (servings/d) of margarine products, eggs, fatty fish, lean fish, red meat, poultry, high-fat dairy, and low-fat dairy. Analysis of covariance was used to obtain mean 25(OH)D concentrations with adjustment for sex, age, and season according to tertiles of body fatness. $P$ values for interaction were obtained by adding a multiplicative interaction term to the regression model. All reported $P$ values were two-sided, and $P$ values $<0.05$ were considered statistically significant.

**RESULTS**

The characteristics of the study population are shown in Table 1. Approximately 50% of the population were women, and the mean (±SD) age was 69.6 ± 6.5 y. In the winter period, 51% had a 25(OH)D concentration <50.0 nmol/L compared with 34% in the summer period (Table 2).

**Determinants of 25-hydroxyvitamin D concentrations**

After multivariable adjustment, older age, higher educational level, and higher body fatness were significantly associated with worse vitamin D status (Table 3). More time spent on outdoor activities, use of vitamin D–containing supplements, and consumption of margarine products (vitamin D fortified in the Netherlands), fatty fish, and red meat were associated with better vitamin D status. In contrast, cigarette smoking, and consumption of dairy products (not fortified), eggs, lean fish, and poultry were not substantially associated with vitamin D status. When calcium intake was included in the multivariable model instead of dairy products, it was not associated with higher 25(OH)D concentrations either ($\beta$ [±SE] calcium intake: $-0.32 ± 0.24$ nmol/L per 100 mg/d increment; $P = 0.18$).

**Sex differences in vitamin D status**

After adjustment for only age and season, women had a 6.0 ± 1.5 nmol/L lower 25(OH)D concentration than did the men (Table 3). After adjustment for percentage body fat, this association disappeared (women compared with men: $-0.2 ± 2.2$ nmol/L), which suggests that the greater body fatness of women explains the sex difference in vitamin D status. As additionally indicated in Figure 1, the vitamin D status of men and women did not differ significantly at the same body fat percentage. The association between body fatness and vitamin D status did not differ substantially by sex ($P$ for interaction = 0.38).
TABLE 1
Characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Men (n = 271)</th>
<th>Women (n = 267)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>69.4 ± 6.3</td>
<td>69.8 ± 6.7</td>
<td>0.43</td>
</tr>
<tr>
<td>Percentage body fat (%)</td>
<td>27.6 ± 6.5</td>
<td>41.5 ± 6.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Current cigarette smoker [n (%)]</td>
<td>55 (20.3)</td>
<td>31 (11.6)</td>
<td>0.006</td>
</tr>
<tr>
<td>Low education level [n (%)]</td>
<td>117 (43.2)</td>
<td>140 (52.4)</td>
<td>0.03</td>
</tr>
<tr>
<td>Daily use of supplements with vitamin D [n (%)]</td>
<td>27 (10.0)</td>
<td>42 (15.7)</td>
<td>0.05</td>
</tr>
<tr>
<td>Outdoor activities (h/d)</td>
<td>0.9 (0.3–1.8)</td>
<td>0.7 (0.3–1.3)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Differences by sex were evaluated by using Student’s *t* test for continuous variables, the Wilcoxon rank-sum test for continuous variables that were not normally distributed, and a chi-square test for categorical variables.

2 *x ± SD* (all such values).
3 Low level denotes elementary school, lower vocational training, or less.
4 Median; interquartile range in parentheses (all such values).
5 Serving sizes were 10 g for butter and cheese and 150 g for fluid dairy products.

Margarine, fatty fish, and supplement use in relation to vitamin D inadequacy

Because consumption of foods and use of supplements are often considered as targets for interventions to achieve adequate vitamin D status in populations, we further examined these factors in relation to vitamin D inadequacy. On the basis of the strength of the associations with plasma 25(OH)D concentrations, we limited this analysis to the use of margarine products, fatty fish, and vitamin D–containing supplements. All these factors were inversely associated with vitamin D inadequacy (Table 4). However, the prevalence of vitamin D inadequacy was still substantial for participants with favorable levels for these exposures (Table 4). We also examined combinations of margarine, fish, and supplement use. No suggestions of interactions were observed between margarine, fatty fish, and supplement use in relation to 25(OH)D concentrations or prevalence of vitamin D inadequacy (all P values for interaction > 0.40). From the multivariable-adjusted regression coefficients, we estimated that the combined use of 20 g margarine products/d, ≥1 vitamin D–containing supplement/d, and 100 g fatty fish/wk (a total intake of ≈6.4 μg/d vitamin D; Table 5) was associated with a 16.8 nmol/L higher 25(OH)D concentration and an odds ratio of vitamin D inadequacy of 0.07. However, none of the participants reached these intakes for all 3 factors.

TABLE 2
Vitamin D status of the study population based on plasma 25-hydroxyvitamin D [25(OH)D] concentrations in 538 Dutch men and women aged 60–87 y

<table>
<thead>
<tr>
<th></th>
<th>Summer months (n = 172)</th>
<th>Winter months (n = 366)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D concentration (nmol/L)</td>
<td>61.3 ± 20.1a</td>
<td>50.5 ± 18.3b</td>
</tr>
<tr>
<td>25(OH)D percentiles (nmol/L)</td>
<td>5th</td>
<td>23.8</td>
</tr>
<tr>
<td></td>
<td>25th</td>
<td>35.3</td>
</tr>
<tr>
<td></td>
<td>50th</td>
<td>49.0</td>
</tr>
<tr>
<td></td>
<td>75th</td>
<td>64.0</td>
</tr>
<tr>
<td></td>
<td>95th</td>
<td>84.1</td>
</tr>
<tr>
<td>25(OH)D categories [n (%)]</td>
<td>&lt;25.0 nmol/L</td>
<td>3 (1.7)</td>
</tr>
<tr>
<td></td>
<td>25.0–49.9 nmol/L</td>
<td>55 (32.0)</td>
</tr>
<tr>
<td></td>
<td>50.0–74.9 nmol/L</td>
<td>73 (42.4)</td>
</tr>
<tr>
<td></td>
<td>≥75.0 nmol/L</td>
<td>41 (23.8)</td>
</tr>
</tbody>
</table>

* Differences from summer months, *P* < 0.001 (Student’s *t* test for continuous variables and chi-square test for categorical variables).

1 Summer months: June through November; winter months: December through May.
2 *x ± SD* (all such values).
3 Significantly different from summer months, *P* < 0.001 (Student’s *t* test for continuous variables and chi-square test for categorical variables).

DISCUSSION

In our population-based study of older white men and women in the Netherlands, lower body fatness, more time spent on outdoor physical activity, and use of fortified margarine products, fatty fish, and vitamin D–containing supplements were associated with better vitamin D status. Individually, however, regular use of vitamin D–containing supplements, fatty fish, and fortified margarine were not sufficient to achieve adequate vitamin D status.

Older age and female sex were associated with substantially worse vitamin D status, which agrees with results from earlier studies (8, 15, 16). Our data suggests that the sex difference in vitamin D status may be due to the generally higher body fatness observed in women than in men. An inverse association between body fatness and vitamin D status has been reported previously (16–18) and may reflect excess vitamin D storage in adipose
It was also suggested that poor vitamin D status may increase adiposity through increased lipogenesis as a result of elevated parathyroid hormone concentrations (17).

Consistent with previous studies, regular use of fatty fish (8, 9) or vitamin D–containing supplements (11, 15, 20, 21) were associated with substantially better vitamin D status than was no

**TABLE 3**
The association between dietary and other potential predictors of vitamin D status and plasma 25-hydroxyvitamin D [25(OH)D] concentrations in 538 Dutch men and women aged 60–87 y

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Increment</th>
<th>Adjusted for age, sex, and season¹</th>
<th></th>
<th></th>
<th>Adjusted for other predictors²</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>10 y</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Female vs male</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Body fat</td>
<td>10%</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Cigarette smoker</td>
<td>Current vs never</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Education level²</td>
<td>Medium vs low</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Supplements with vitamin D</td>
<td>Daily use vs no use</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Outdoor activities</td>
<td>1 h/d</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Food consumption</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Margarine products</td>
<td>1 serving/d</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Eggs</td>
<td>1 serving/d</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Fatty fish</td>
<td>1 serving/wk</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Lean fish</td>
<td>1 serving/wk</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Red meat</td>
<td>1 serving/wk</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Poultry</td>
<td>1 serving/wk</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>High-fat dairy</td>
<td>1 serving/d</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Low-fat dairy</td>
<td>1 serving/d</td>
<td>RC (SE)</td>
<td>P</td>
<td>RC (SE)</td>
<td>P</td>
<td></td>
</tr>
</tbody>
</table>

¹ Adjusted for age (y), sex, and season (December through February, March through May, June through August, or September through November) by using multivariable linear regression analysis. Additional adjustment for total energy intake (kJ/d) was performed for the models with food variables.

² The multivariable model included age, sex, season, total energy intake, body fat (%), cigarette smoking (current, past, or never), education level (high, medium, or low), use of vitamin D–containing supplements (none, <1/d, or ≥1/d), outdoor activities (h/d), and consumption (servings/d) of margarine products, eggs, fatty fish, lean fish, red meat, poultry, high-fat dairy, and low-fat dairy.

³ Regression coefficient (RC) reflecting the difference in 25(OH)D (nmol/L) for each given increment in the exposure variable. Serving sizes were 10 g for margarine, butter, and cheese; 150 g for fluid dairy products; 100 g for red meat, poultry, and fish; and 50 g for eggs.

⁴ High level includes vocational college or university, medium level includes secondary education, and low level includes elementary school, lower vocational training, or less.

*FIGURE 1.* Mean (±SE) plasma 25-hydroxyvitamin D [25(OH)D] concentrations according to sex-specific tertiles of percentage body fat in 538 Dutch men (♦) and women (■) aged 60–87 y. Data were adjusted for age, season, and educational level by using ANCOVA.
use of these products. Except for fatty fish, however, other foods with naturally occurring vitamin D contributed little to vitamin D status, consistent with their low vitamin D content (Table 5). Consumption of vitamin D–fortified margarine was associated with better vitamin D status in our study, whereas consumption of dairy products that are not fortified in the Netherlands was not. Similarly, intake of calcium from vitamin D–fortified, but not from nonfortified, sources was associated with substantially better vitamin D status in a study conducted in the United States (22). A high calcium intake may have a vitamin D–sparing effect because of a decrease in serum parathyroid hormone and decreased turnover of vitamin D metabolites (2), but this effect may be less relevant in populations with a high calcium intake such as our study population (average intake: 1054 mg/d for men and 1065 mg/d for women). A longitudinal study conducted in young Finnish men suggested that fortification of milk and margarine with vitamin D reduced the prevalence of vitamin D inadequacy by 50%, also underscoring the importance of appropriate vitamin D fortification (23). The Dutch Commodities Act does not allow the addition of vitamin D to foods other than margarine products, with the exception of small amounts for restoration or for substitution products (Table 5). However, in 2004 the European Court of Justice ruled that the Netherlands cannot generally prohibit the addition of vitamin D to foods but has to consider applications for the addition of vitamin D on a case-by-case basis. Studies conducted in the Netherlands and the United Kingdom have shown a much higher prevalence of vitamin D deficiency in persons of non-Western origin than in other residents (4, 5). Because margarine products are less likely to be used (24) and lactose intolerance is more prevalent in these high-risk groups, fortification of foods other than margarine and milk with vitamin D is preferable.

### Table 4

<table>
<thead>
<tr>
<th>Categories of exposure</th>
<th>n</th>
<th>25(OH)D &lt;50.0 nmol/L</th>
<th>OR (95% CI)</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Margarine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No use</td>
<td>59</td>
<td>34 (58)</td>
<td>1 (ref)</td>
<td></td>
</tr>
<tr>
<td>&lt;10 g/d</td>
<td>155</td>
<td>89 (57)</td>
<td>0.83 (0.40, 1.74)</td>
<td></td>
</tr>
<tr>
<td>10–19.9 g/d</td>
<td>163</td>
<td>70 (43)</td>
<td>0.55 (0.27, 1.14)</td>
<td></td>
</tr>
<tr>
<td>≥20 g/d</td>
<td>161</td>
<td>51 (32)</td>
<td>0.41 (0.20, 0.86)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fatty fish</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No use</td>
<td>69</td>
<td>43 (62)</td>
<td>1 (ref)</td>
<td></td>
</tr>
<tr>
<td>&lt;1.0 serving/mo</td>
<td>287</td>
<td>130 (46)</td>
<td>0.66 (0.34, 1.30)</td>
<td></td>
</tr>
<tr>
<td>1.0–1.9 servings/mo</td>
<td>102</td>
<td>38 (38)</td>
<td>0.47 (0.21, 1.04)</td>
<td></td>
</tr>
<tr>
<td>≥2.0 servings/mo</td>
<td>86</td>
<td>33 (38)</td>
<td>0.41 (0.16, 1.04)</td>
<td>0.01</td>
</tr>
<tr>
<td>Vitamin D–containing supplements</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No use</td>
<td>460</td>
<td>220 (48)</td>
<td>1 (ref)</td>
<td></td>
</tr>
<tr>
<td>&lt;1/d</td>
<td>9</td>
<td>2 (22)</td>
<td>0.55 (0.09, 3.33)</td>
<td></td>
</tr>
<tr>
<td>≥1/d</td>
<td>69</td>
<td>22 (32)</td>
<td>0.33 (0.17, 0.63)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

1 Vitamin D inadequacy was defined as a plasma 25-hydroxyvitamin D [25(OH)D] concentration <50 nmol/L.

2 Multivariate-adjusted (as described in the footnotes to Table 2) by using logistic regression analysis.

3 Calculated by modeling the exposures as continuous variables.

### Table 5

**Vitamin D content of selected Dutch foods and supplements**

<table>
<thead>
<tr>
<th>Food</th>
<th>Serving size</th>
<th>Concentration vitamin D (per serving)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplements</td>
<td>1</td>
<td>≤5.0 μg</td>
<td>Amount allowed per daily dose (typically 2.5 or 5.0 μg per supplement)</td>
</tr>
<tr>
<td>Supplements for “at-risk groups”</td>
<td>1</td>
<td>≤15.0 μg</td>
<td>Label should target children aged &lt;6 y or pregnant or lactating women. Since 2005, also allowed for persons aged ≥60 y²</td>
</tr>
<tr>
<td>Margarine, half-fat</td>
<td>10 g</td>
<td>0.72 μg</td>
<td>Fortified as agreed in a covenant between government and industry with a maximum of 0.075 μg/g³</td>
</tr>
<tr>
<td>Butter</td>
<td>10 g</td>
<td>0.12 μg</td>
<td>Not fortified</td>
</tr>
<tr>
<td>Full-fat milk</td>
<td>150 mL</td>
<td>0.15 μg</td>
<td>Not fortified</td>
</tr>
<tr>
<td>Reduced-fat milk (2%)</td>
<td>150 mL</td>
<td>0.00 μg</td>
<td>Not fortified</td>
</tr>
<tr>
<td>Mackerel, smoked</td>
<td>100 g</td>
<td>8.0 μg</td>
<td>Not fortified</td>
</tr>
<tr>
<td>Salmon, microwave-cooked</td>
<td>100 g</td>
<td>8.7 μg</td>
<td>Not fortified</td>
</tr>
</tbody>
</table>

1 Values for the foods were calculated from data in reference 14.

2 Dutch Commodities Act Regulation Exemption for vitamin preparations.

3 If the label explicitly targets persons aged ≥60 y, fortification of 0.20–0.25 μg/g margarine product is allowed since 2003 (Dutch Commodities Act Regulation Exemption for vitamin D), but this type of margarine does not seem to be commonly available.
vitamin D toxicity (25). Vitamin D intoxication can lead to hypercalcaemia, hypercalciuria, bone resorption, bone loss, and impairment of renal function (2). However, hypercalcaemia due to effects of vitamin D intoxication per se has only been observed for 25(OH)D concentrations >220 nmol/L (25), whereas the 95th percentile concentration observed in our study population for the summer period was 99 nmol/L. This suggests that vitamin D intakes can be substantially higher before toxicity becomes a concern and that risk of vitamin D intoxication is negligible with regular fortification policies (eg 10 µg of vitamin D/L milk or orange juice) (25).

The associations of season and outdoor physical activity with vitamin D status are consistent with the importance of cutaneous vitamin D production under the influence of sunlight. Previous studies have also linked a tendency to stay indoors (8, 15) and wearing long sleeved clothing in sunshine (8) to poor vitamin D status. Although excessive exposure to sunlight increases the risk of skin cancer, sensible sun exposure without getting burned (usually 5–10 min of exposure of the arms and legs or of the hands, arms, and face 2 or 3 times/wk) may be prudent for improving vitamin D status (6). However, for many populations, it seems unlikely that increased sun exposure is a sufficient remedy for inadequate vitamin D status. Poor vitamin D status is observed in countries with abundant sunlight (3), and at the latitude of the Netherlands (≈52°N) solar radiation is sufficient for vitamin D formation in only 6 mo of the year (26). Therefore, adequate vitamin D stores have to be built up in the summer to prevent vitamin D deficiency in the winter, but in practice, this usually is not sufficient. Also, for the elderly and persons with modest dress (that leaves little of the skin uncovered) because of religious or cultural reasons, it can be difficult to increase sun exposure sufficiently to reach an adequate vitamin D status (24).

Although the direction of effects cannot be determined in cross-sectional studies, an effect of vitamin D status on lifestyle factors does not seem plausible, because the participants were unlikely to be aware of their vitamin D status. Because our study was not a randomized trial, we cannot exclude the possibility of confounding by imperfectly measured or unmeasured factors. In addition, assessment of lifestyle factors by using self-reports has undoubtedly led to some measurement error and a reduced ability to detect associations for contributors of small amounts of vitamin D. The observed associations are generally consistent, however, with data from intervention studies (3, 25) and the vitamin D content of foods and supplements (Table 5).

Our findings suggest that increased adiposity and a sedentary lifestyle, which result in less participation in outdoor activities, may contribute to poor vitamin D status. Because few foods are vitamin D fortified and amounts of vitamin D in supplements are low, it is difficult to achieve adequate vitamin D status through increasing intakes in the Netherlands and countries with similar policies. Use of supplements with higher vitamin D doses would be an effective measure for specific high-risk groups, but the experience from campaigns recommending folate supplements suggest that this strategy may not be effective for large parts of the general population (27). Our results indicate that fortification of margarine with vitamin D substantially contributes to better vitamin D status in the Netherlands and that fortification of other widely used foods, such as milk, yogurt, orange juice (28), and cereal products (29), should be considered.

RMvD conceived the research question, conducted the statistical data analysis, and drafted the manuscript. MBS, JMD, CDAS, LMB, and RJJ were involved in the data collection. All authors advised on interpretation of the results, revised the paper critically, and approved the final manuscript. PL received funds for research, fees for consulting, or both from Nycome, Lilly, MSD, Wyeth, Servier, Aventis, and Procter & Gamble. None of the other authors had a personal or financial conflict of interest.

REFERENCES

22. Kinyamu HK, Gallagher JC, Rafferty KA, Balhorn KE. Dietary calcium


Effect of dietary lutein and zeaxanthin on plasma carotenoids and their transport in lipoproteins in age-related macular degeneration

Wei Wang, Sonja L Connor, Elizabeth J Johnson, Michael L Klein, Shannon Hughes, and William E Connor

ABSTRACT

Background: Low dietary intakes and low plasma concentrations of lutein and zeaxanthin are associated with an increased risk of age-related macular degeneration (AMD). No studies have challenged AMD patients with a diet high in lutein and zeaxanthin.

Objective: The objective was to examine the effect of diets low or high in lutein and zeaxanthin on plasma carotenoids and their transport in AMD patients.

Design: Seven AMD patients and 5 control subjects were fed a low-lutein, low-zeaxanthin diet (=1.1 mg/d) for 2 wk, which was followed by a high-lutein, high-zeaxanthin diet (=11 mg/d) for 4 wk. Ten subjects continued the diet for 8 wk. Plasma and lipoprotein carotenoids were measured by HPLC.

Results: The high-lutein, high-zeaxanthin diet resulted in 2- to 3-fold increases in plasma concentrations of lutein and zeaxanthin and other carotenoids, except lycopene, in the AMD patients and the control subjects. With this diet, 52% of the lutein and 44% of the zeaxanthin were transported by HDL; 22% of lutein and zeaxanthin was transported by LDL. Only 20–25% of α-carotene, β-carotene, and lycopene was transported by HDL; 50–57% was transported by LDL.

Conclusions: The AMD patients and control subjects responded similarly to a diet high in lutein and zeaxanthin; plasma carotenoid concentrations increased greatly in both groups, and the transport of carotenoids by lipoproteins was not significantly different between the groups. This finding suggests that abnormalities in the metabolism of lutein and zeaxanthin in AMD may reside in the uptake of lutein and zeaxanthin from the plasma and transport into the retina.


KEY WORDS HDL cholesterol, LDL cholesterol, VLDL cholesterol, retina, age, macular degeneration

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in persons aged ≥65 y and affects >6 million people in the United States (1, 2). Of the many nutritional factors associated with the risk of developing advanced AMD is a reduced dietary intake of the carotenoids lutein and zeaxanthin (3, 4). Lutein and zeaxanthin intakes of =6 mg/d or greater have been related to a decreased risk of AMD (3). The typical US diet contains ≈1–3 mg/d of lutein and zeaxanthin combined (3–6).

The dietary intake of lutein and zeaxanthin has been shown to influence the plasma concentrations of these carotenoids and their content in the macula in humans without AMD and in nonhuman primates (7–16). The macula of monkeys given a lutein- and zeaxanthin-free diet had more oxidative damage than did the macula of monkeys fed a diet containing these carotenoids (10). Macular pigments were not detected in primates who were fed lutein- and zeaxanthin-deficient diets. However, feeding lutein and zeaxanthin to the deficient monkeys led to some restoration of macular pigments (11, 14, 15).

Healthy human subjects given a lutein supplement, either in the form of lutein tablets or of supplemented foods, resulted in significant increases in plasma lutein concentrations (7–9, 12, 13, 16). Macular pigment density was significantly correlated with plasma concentrations and dietary intakes of lutein and zeaxanthin, although the response varied among subjects (8, 9). Donor eyes of AMD patients had a significantly lower content of lutein and zeaxanthin than did donor eyes with normal retinas (17).

Carotenoids are lipophilic plant pigments that are transported by lipoproteins in the plasma of humans and animals (18). LDL is the primary transporter of carotenes; HDL is the primary transporter of xanthophylls such as lutein and zeaxanthin. The lipoprotein transport of carotenoids in AMD patients has not been studied extensively. A recent report showed that fasting carotenoid concentrations and their distribution in lipoproteins were not significantly different between AMD patients and control subjects (19).

The objective of this study was to determine whether differences exist between AMD patients and control subjects in their plasma carotenoid responses to a diet high in lutein and zeaxanthin and in their transport of lutein and zeaxanthin. We challenged AMD patients and control subjects with a diet of whole foods high in lutein and zeaxanthin after a control period of a diet low in lutein and zeaxanthin. No previous studies of the effects of lutein and zeaxanthin from the plasma and transport into the retina. This finding suggests that abnormalities in the metabolism of lutein and zeaxanthin may reside in the uptake of lutein and zeaxanthin from the plasma and transport into the retina.
high-lutein, high-zeaxanthin diets on plasma carotenoid responses have been conducted in patients with AMD. Only a few studies of such effects have been conducted in healthy subjects, who showed a response to both supplements and to a diet high in lutein and zeaxanthin.

**SUBJECTS AND METHODS**

**Subjects**

Seven patients with advanced AMD with greatly reduced vision in one eye and good visual acuity (20/30 or better) in the fellow eye were recruited from the Casey Eye Institute, Oregon Health and Science University (OHSU). Five control subjects of similar ages without signs of AMD in either eye were either spouses of the AMD patients or were recruited from other OHSU clinics (Table 1). Macular status was assessed by using the Age-Related Eye Disease Study System (AREDS) to classify AMD: category 1, no macular abnormality in either eye; category 2, mild or borderline macular abnormality or AMD features; category 3, many small or few intermediate drusen or pigment abnormalities; and category 4, advanced AMD in at least one eye (20). All subjects were non-Hispanic whites. Both the AMD patients and the control subjects were elderly and had similar disease and biochemical characteristics other than AMD diagnosis. The study protocol was approved by the OHSU Institutional Review Board, and the subjects provided informed consent before the study began.

**Study design**

The overall design of the study is shown in Figure 1. Initially, the number of subjects was based on published data indicating that the 2 groups would be equal and our expectation was that the change in the AMD group would be only 50% of that of the control group. Power was estimated at 74% with a sample size of 18 subjects per group (total of 36 subjects). An interim analysis was carried out in 12 subjects, which indicated that, to achieve significance ($P < 0.05$), a difference in the changes between the AMD and control groups in plasma lutein with the 2 diets would have to be 8.1 $\mu$g/dL on the basis of the observed SDs of the changes in the 12 subjects. This implies that the difference in changes for the remaining 24 subjects would need to exceed 10.0 $\mu$g/dL to attain a power of 74%, which is highly unlikely given...
that the observed difference in the first one-third of the study was only 2.5 μg/dL. This, coupled with the considerable amount of time and effort involved on the part of the subjects to participate in the 12-wk highly controlled feeding study, led to the decision to end the study with 12 subjects.

Diets

There were 2 dietary phases: a typical low-lutein, low-zeaxanthin diet (2 wk) and a high-lutein, high-zeaxanthin diet (12 wk). All food was prepared for the subjects for the first 6 wk of the study. The subjects were instructed to prepare a diet high in lutein and zeaxanthin for the last 8 wk. All diets met energy and other nutritional needs.

The diet low in lutein and zeaxanthin (~1100 μg/d, or 1.1 mg/d) and other carotenoids was fed for 2 wk. The low-lutein, low-zeaxanthin diet was similar to a typical US diet with regard to carotenoids, protein, fat, and carbohydrate (4, 21). The diet high in lutein and zeaxanthin (~11 000 μg/d, or 11 mg/d—10 times the amount in the low diet) was fed for 4 wk. Six AMD patients and 4 control subjects continued the high-lutein, high-zeaxanthin diet for an additional 8 wk. The subjects prepared the diet at home with considerable guidance and monitoring by registered dietitians. The subjects kept daily records of the amounts of foods that they consumed.

The diet high in lutein and zeaxanthin had a high content of fruit and vegetables and was lower in fat and higher in complex carbohydrate and fiber than was the diet low in lutein and zeaxanthin. Examples of one day of foods for each diet are given in Table 2. Because of the concern for a spillover effect on plasma and lipoprotein carotenoid concentrations from the high-carotenoid diet, the diets were not randomized; the diet low in lutein and zeaxanthin was fed first. A multivitamin containing 250 μg lutein was provided daily to all subjects, because this was the amount typically consumed by the subjects before they entered the study.

For the first 2 wk of the diet low in lutein and zeaxanthin and for the following 4 wk of the diet high in lutein and zeaxanthin, all meals were prepared by the General Clinical Research Center (GCRC), Bionutrition Department, OHSU. The subjects came to the GCRC 3 times/wk to be weighed and to eat breakfast. Foods were then packaged for the remainder of the day and the following day. On Fridays, foods were packaged for the weekend. All uneaten foods were returned to the GCRC and were weighed. Daily nutrient intakes were computed for each subject on the basis of the foods consumed. Nutrient calculations were performed by using the Nutrition Data System for Research (NDS-R, software version 4.02, Food and Nutrient Database 30, released November 1999; Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN) (22).

Plasma lipids and lipoproteins

Fasting plasma samples were drawn weekly for 6 wk when food was provided and twice at 4-wk intervals during the home meal preparation. Because a 30-mg lutein, 50-g fat tolerance test was done at the beginning of the last week of each diet period, to minimize any effect of the test on carotenoid distributions in lipoproteins, data were used from the end of week 1 of the diet low in lutein and zeaxanthin and from the end of week 3 of the diet high in lutein and zeaxanthin. The data used in Figure 2 were from the end of weeks 1 and 2 of the typical low-lutein diet and weeks 1, 2, 3, 4, 8, and 12 of the high-lutein diet.

Blood was collected into tubes containing EDTA. Plasma was immediately centrifuged in polypropylene tubes by sequential
Plasma and lipoprotein lutein, zeaxanthin, and other carotenoids

Plasma and lipoprotein carotenoids were measured as described previously (13). Briefly, plasma and lipoprotein fraction samples were protected from light and stored at −80 °C until analyzed. Echinone in ethanol was added as an internal standard to 200 μL serum or lipoprotein fraction sample and 0.5 mL of 0.9% saline. The mixture was extracted by using 2 mL chloroform:methanol (2:1, by vol). The mixture was mixed by vortex and centrifuged and the chloroform layer was removed. A second extraction was done on the mixture with the use of 3 mL hexane, which was followed by mixing by vortex and centrifugation. The hexane layer was combined with the first extraction and evaporated to dryness under nitrogen. The residue from serum was redissolved in 150 μL ethanol, mixed by vortex, and sonicated for 30 s. A 50-μL aliquot was used for the HPLC analysis (16).

A C30 carotenoid column was used for the carotenoid measurements. Carotenoids were quantified by determining peak areas in the HPLC chromatograms and calibrated against known amounts of standards. Carotenoid standards were provided by Roche Vitamins, Ltd (now DSM Nutritional Products, Parsippany, NJ). The percentage of carotenoids in each lipoprotein fraction was calculated as the absolute concentration of each fraction divided by the sum of the concentrations of all fractions. In our ultracentrifugation procedures, all the lipoprotein fractions to total plasma analyte concentration). Statistical analyses

Results are expressed as means (±SEM). Data were assessed by using 2-factor (group and diet) repeated-measures analysis of variance (SPSS, version 14.0). If the interaction (group × diet) was not significant, no subgroup analysis was performed (the effect due to time was made across groups). If a significant interaction was found, a subgroup analysis was performed with a Bonferroni correction. Differences were considered statistically significant at P < 0.05.

RESULTS

Diets

Nutrient intakes were computed from actual dietary intakes for the periods in which all food was provided. Because no significant differences in the nutrient intakes during each dietary period were found between the AMD and control groups, the data were combined (Table 3). The subjects consumed ~10 times more lutein and zeaxanthin with the diet high in lutein and zeaxanthin

TABLE 3

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Low L/Z diet (n = 12)</th>
<th>High L/Z diet (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lutein + zeaxanthin</td>
<td>1101 ± 27</td>
<td>11 211 ± 6452</td>
</tr>
<tr>
<td>Beta-Cryptoxanthin</td>
<td>87 ± 6</td>
<td>473 ± 25</td>
</tr>
<tr>
<td>Alpha-Carotene</td>
<td>246 ± 18</td>
<td>827 ± 215</td>
</tr>
<tr>
<td>Beta-Carotene</td>
<td>1259 ± 44</td>
<td>6793 ± 391</td>
</tr>
<tr>
<td>Lycopene</td>
<td>6400 ± 413</td>
<td>7504 ± 593</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>2127 ± 80</td>
<td>1984 ± 103</td>
</tr>
<tr>
<td>Fat (% of energy)</td>
<td>34 ± 0</td>
<td>24 ± 12</td>
</tr>
<tr>
<td>Saturated fat (% of energy)</td>
<td>11 ± 0</td>
<td>7 ± 12</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>214 ± 8</td>
<td>117 ± 11</td>
</tr>
<tr>
<td>Carbohydrate (% of energy)</td>
<td>53 ± 1</td>
<td>62 ± 12</td>
</tr>
<tr>
<td>Fiber (mg)</td>
<td>23 ± 1</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>Retinol (μg)</td>
<td>493 ± 28</td>
<td>501 ± 29</td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>12 ± 0</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>65 ± 3</td>
<td>202 ± 6</td>
</tr>
</tbody>
</table>

1 All values are ± SEM. There were no significant group-by-diet interactions by 2-factor repeated-measures ANOVA. 2 Significant differences were observed between the low and high L/Z diets in both groups combined, P < 0.05 (paired t test).
than with the diet low in lutein and zeaxanthin. Except for lycopene, the subjects also consumed 3–5 times the amount of all other carotenoids because of their presence in the same foods that are high in lutein and zeaxanthin. Lycopene is found primarily in tomato products, foods that were not increased in the diet high in lutein and zeaxanthin. With the diet high in lutein and zeaxanthin, the subjects also consumed fewer calories; less saturated fat, total fat, and cholesterol; and more carbohydrate, fiber, and vitamin C. Retinol and vitamin E intakes were not significantly different between groups.

### Plasma lutein and zeaxanthin concentrations

Plasma lutein was greater in both the AMD and control groups during the diet high in lutein and zeaxanthin than during the diet low in lutein and zeaxanthin. The concentrations remained elevated throughout the 12 wk of the diet high in lutein and zeaxanthin (Figure 2). The rapidity of the increase in plasma lutein was of interest. One week after the diet high in lutein and zeaxanthin, the plasma lutein concentration had increased significantly and doubled after 2 wk. The downward drift in plasma lutein in the control group at weeks 8 and 12 occurred because 2 subjects were unable to maintain their earlier intakes of lutein and zeaxanthin; home diets were assessed from food records.

The response of the AMD and control subjects was not significantly different, and no significant group-by-time interaction was observed (Table 4). After 2 wk of the diet low in lutein and zeaxanthin (≈1.1 mg/d), plasma lutein was 12.79 ± 1.65 μg/dL for the control group and 9.91 ± 1.36 μg/dL for the AMD group. Likewise, plasma zeaxanthin was 2.09 ± 0.38 μg/dL for the control group and 1.94 ± 0.36 μg/dL for the AMD group. The main effect of time was significantly different for both the AMD and control groups (P < 0.05). Four weeks of the diet high in lutein and zeaxanthin resulted in a 2- to 3-fold increase in plasma lutein in both the control (26.07 ± 4.72 μg/dL) and AMD (25.72 ± 4.00 μg/dL) groups. Plasma zeaxanthin was significantly greater in both the AMD (3.57 ± 0.66 μg/dL) and the control (3.57 ± 0.87 μg/dL) groups at week 4 of the diet high in lutein and zeaxanthin than at week 2 of the diet low in lutein and zeaxanthin. Two control subjects had difficulty eating the high-lutein diet that was prepared for them.

Considering lutein and zeaxanthin together, the plasma was ≈80% lutein and 20% zeaxanthin with the diet low in lutein and zeaxanthin (lutein:zeaxanthin = 4:1) and 87% lutein and 13% zeaxanthin with the diet high in lutein and zeaxanthin (lutein:zeaxanthin = 6.7:1). This is comparable with the chemical analysis of the 1 d of food in which lutein was 87% and zeaxanthin was 13% for both the diets low and high in lutein and zeaxanthin (lutein:zeaxanthin = 6.7:1).

### Other plasma carotenoids

Concentrations of β-cryptoxanthin, α-carotene, β-carotene, and lycopene in plasma, LDL, and HDL after 1 wk of the diet low in lutein and zeaxanthin and after 3 wk of the diet high in lutein and zeaxanthin are shown in Table 5. No significant group-by-diet interaction was observed, except for plasma and LDL β-carotene. Although the dietary intake increased ≈5-fold in both groups, β-carotene increased significantly only in the control subjects. This finding was likely due to the fact that the AMD patients started out with high plasma concentrations of β-carotene. With the diet low in lutein and zeaxanthin, plasma β-carotene was significantly greater in the AMD group (35.75 μg/dL) than in the control group (15.14 μg/dL). Even though the mean dietary intake increased significantly from 1 to 7 mg, no significant increase in plasma β-carotene was observed in the AMD group. Plasma β-carotene increased significantly in all 5 control subjects but in only 1 AMD patient. In the AMD group, it remained unchanged in 4 subjects and decreased in 2 subjects, who had been taking 17 mg/d β-carotene as a supplement daily before the study, but who did not take it during the study.

Plasma β-cryptoxanthin and α-carotene were significantly greater with the diet high in lutein and zeaxanthin than with the diet low in lutein and zeaxanthin in both groups. Plasma lycopene concentrations were not significantly different between the diets or groups.

### Distribution of carotenoids among lipoproteins

The concentrations of the major carotenoids in plasma, LDL, and HDL after both diets are shown in Table 5. The distribution of carotenoids among lipoproteins was not significantly different between the AMD and control subjects.
Lutein, zeaxanthin, β-cryptoxanthin, and α-carotene concentrations in the VLDL fraction were significantly greater with the diet high in lutein and zeaxanthin than with the diet low in lutein and zeaxanthin in both groups (data not shown). β-Carotene in the VLDL fraction was significantly greater with the diet high in lutein and zeaxanthin than with the diet low in lutein and zeaxanthin in both groups.

TABLE 5
Concentrations of the major carotenoids in plasma, LDL, and HDL after the consumption for 1 wk of the diet low (≈1.1 mg/d) and for 3 wk of the diet high (≈11 mg/d) in lutein and zeaxanthin (L/Z) diet in the patients with age-related macular degeneration (AMD) and the control subjects.

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>LDL</th>
<th>HDL</th>
<th>HDL:LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low L/Z diet</td>
<td>High L/Z diet</td>
<td>Low L/Z diet</td>
<td>High L/Z diet</td>
</tr>
<tr>
<td>Control subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lutein (µg/dL)</td>
<td>8.35 ± 2.04</td>
<td>27.53 ± 5.67</td>
<td>1.56 ± 0.51</td>
<td>5.93 ± 1.60</td>
</tr>
<tr>
<td>Zeaxanthin (µg/dL)</td>
<td>2.08 ± 0.55</td>
<td>3.66 ± 1.05</td>
<td>0.54 ± 0.18</td>
<td>1.03 ± 0.26</td>
</tr>
<tr>
<td>β-Cryptoxanthin (µg/dL)</td>
<td>5.53 ± 1.09</td>
<td>16.05 ± 3.32</td>
<td>2.33 ± 0.37</td>
<td>4.76 ± 1.35</td>
</tr>
<tr>
<td>α-Carotene (µg/dL)</td>
<td>2.46 ± 0.59</td>
<td>6.34 ± 1.96</td>
<td>1.62 ± 0.40</td>
<td>4.85 ± 2.51</td>
</tr>
<tr>
<td>β-Carotene (µg/dL)</td>
<td>15.14 ± 5.50</td>
<td>36.18 ± 7.65</td>
<td>8.72 ± 2.16</td>
<td>22.69 ± 5.57</td>
</tr>
<tr>
<td>Lycopene (µg/dL)</td>
<td>15.17 ± 2.89</td>
<td>15.26 ± 3.12</td>
<td>12.18 ± 3.93</td>
<td>8.99 ± 1.84</td>
</tr>
<tr>
<td>AMD patients (n = 7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lutein (µg/dL)</td>
<td>8.38 ± 1.35</td>
<td>23.67 ± 5.23</td>
<td>1.51 ± 0.31</td>
<td>4.82 ± 1.11</td>
</tr>
<tr>
<td>Zeaxanthin (µg/dL)</td>
<td>2.12 ± 0.30</td>
<td>3.65 ± 0.73</td>
<td>0.60 ± 0.06</td>
<td>0.82 ± 0.15</td>
</tr>
<tr>
<td>β-Cryptoxanthin (µg/dL)</td>
<td>6.98 ± 1.56</td>
<td>12.98 ± 2.18</td>
<td>2.59 ± 0.70</td>
<td>4.97 ± 1.18</td>
</tr>
<tr>
<td>α-Carotene (µg/dL)</td>
<td>2.73 ± 0.39</td>
<td>3.02 ± 0.57</td>
<td>1.76 ± 0.27</td>
<td>1.40 ± 0.47</td>
</tr>
<tr>
<td>β-Carotene (µg/dL)</td>
<td>35.75 ± 16.57</td>
<td>34.11 ± 12.53</td>
<td>23.99 ± 11.15</td>
<td>18.16 ± 7.74</td>
</tr>
<tr>
<td>Lycopene (µg/dL)</td>
<td>10.99 ± 2.12</td>
<td>17.32 ± 6.57</td>
<td>6.34 ± 1.10</td>
<td>5.70 ± 1.64</td>
</tr>
</tbody>
</table>

1 All values are x ± SEM.
2 Significant main effect of diet across both groups, P < 0.05 (repeated-measures ANOVA).
3 Significantly different from the high L/Z diet, P < 0.05 (repeated-measures ANOVA).
4 Significant diet-by-group interaction, P < 0.05 (repeated-measures ANOVA).

Plasma lipids and lipoproteins and body weight

For all subjects, plasma total and HDL cholesterol were significantly lower with the diet high in lutein and zeaxanthin than with the diet low in lutein and zeaxanthin (total cholesterol: 191 ± 13 and 178 ± 12 mg/dL, P = 0.015; HDL cholesterol: 50 ± 3 and 43 ± 3 mg/dL, P = 0.000), whereas plasma LDL cholesterol and triacylglycerol were not significantly different between diets (LDL cholesterol: 101 ± 10 and 93 ± 10 mg/dL, P = 0.060; triacylglycerol: 158 ± 20 and 163 ± 15 mg/dL, P = 0.648). Body weight was 84.84 ± 5.17 kg with the diet low in lutein and zeaxanthin.
zeaxanthin (week 2) and 83.99 ± 5.02 kg with the diet high in lutein and zeaxanthin (week 4); the difference was not clinically significant ($P = 0.050$).

**DISCUSSION**

The diet high in lutein and zeaxanthin, which provided intakes of 11–12 mg/d, increased plasma lutein concentrations 2- to 3-fold in both the AMD patients and the control subjects. Similar results occurred in 22 healthy subjects fed a high-vegetable diet containing 11 mg lutein and zeaxanthin (12). In another study, healthy subjects fed 12 mg lutein and zeaxanthin daily from spinach and corn had a 2-fold increase in serum lutein (13). Our study indicated that the AMD patients did not respond differently than did the control subjects when challenged with a diet high in lutein and zeaxanthin. The possibility exists that the low statistical power due to a small sample size may have limited the study indicated that the AMD patients did not respond differently to the diet high in carotenoids. These responses were similar to those reported in other studies with intakes of 11–12 mg lutein and zeaxanthin were correlated with the surface area of the lipoproteins. In our studies, the ratio of HDL to LDL was close to 3:1 for lutein and close to 2:1 for zeaxanthin. This ratio was similar in the AMD and control groups and after both the diets low or high in lutein and zeaxanthin. Because HDL-cholesterol concentrations decreased with the diet high in lutein and zeaxanthin, the increase in lutein and zeaxanthin in the total HDL fraction was not caused by an increase in HDL, which actually decreased. Therefore, the surface area of the lipoproteins did not explain the 3:1 and 2:1 HDL-LDL ratios we observed.

The intervention with ≈11 mg lutein and zeaxanthin daily is about twice the recommendation for the prevention of AMD (3), which implies that the lutein and zeaxanthin status of individuals can be readily improved through the consumption of ordinary foods in the diet. The increases in plasma lutein and zeaxanthin were maintained throughout the additional 8 wk of instructed self-feeding at home. It is of interest that both the AMD patients and the control subjects were able to maintain a high intake of vegetables and fruits while preparing their own food. We were impressed at how well these older adults followed the diet high in lutein and zeaxanthin.

There are advantages of lifestyle changes over supplementation, which may have implications for any dietary intervention for AMD. For example, the greater dietary intake of all carotenoids, except lycopene, with the diet high in lutein and zeaxanthin also resulted in increased plasma concentrations of the other carotenoids. These responses were similar to those reported in healthy subjects who were fed a high-vegetable diet (12). Such a diet could have other potential benefits in terms of the prevention of cancer and cardiovascular disease. Supplements of either lutein or zeaxanthin or both without dietary modification would not have these benefits.

Per our chemical analysis, the amounts of lutein and zeaxanthin provided by the diet high in lutein and zeaxanthin were considerably lower than the amounts computed (10.2 mg compared with 14.8 mg) (Table 2). This raises the possibility that the lutein and zeaxanthin intake with the diet high in lutein and zeaxanthin might have been ≈7.5 mg and not ≈11.1 mg. However, there are generally problems associated with both chemical analysis and computer estimates of nutrients. The increases in plasma lutein in our study were comparable with those observed in other studies with intakes of 11–12 mg lutein and zeaxanthin (12, 13) and to a 3-fold increase in plasma lutein and a 2-fold increase in plasma zeaxanthin in one AMD patient and one control subject who were given a supplement containing 12 mg lutein and zeaxanthin (WE Connor, unpublished observations, 2004). Even if the analyzed values were more reflective of actual intake, ≈7.5 mg lutein and zeaxanthin would still be well above the intake of ≈6 mg that is reported to be associated with a reduced risk of AMD (3).

Furthermore, with the diet high in lutein and zeaxanthin, the ratio of lutein to zeaxanthin was similar in the plasma (6.9) and the chemically analyzed aliquot of 1 d of food (6.8). This means that lutein and zeaxanthin were metabolized in the body at similar rates.

HDL was the major lipoprotein transporter of lutein (52%) and zeaxanthin (44%) with the diet high in lutein and zeaxanthin, which was similar to other published data. Clevendon and Bieri (24), who fed healthy young men a typical US diet, showed that 53% of the lutein and zeaxanthin was transported in HDL. Our results also agreed with a recent report that showed no difference in lipoprotein distribution between AMD patients and control subjects; each group had >50% of lutein associated with HDL (19).

Furr and Clark (25) hypothesized that xanthophylls are more likely to be associated with the surface of the lipoproteins, whereas the carotenes more likely exist in the lipid core of the lipoproteins because of their polarity differences. LDL in serum contributes about twice as much total surface lipid as does HDL (18). This should result in a ratio of HDL to LDL of 1:2 if lutein and zeaxanthin were correlated with the surface area of the lipoproteins. In our studies, the ratio of HDL to LDL was close to 3:1 for lutein and close to 2:1 for zeaxanthin. This ratio was similar in the AMD and control groups and after both the diets low or high in lutein and zeaxanthin. Because HDL-cholesterol concentrations decreased with the diet high in lutein and zeaxanthin, the increase in lutein and zeaxanthin in the total HDL fraction was not caused by an increase in HDL, which actually decreased. Therefore, the surface area of the lipoproteins did not explain the 3:1 and 2:1 HDL-LDL ratios we observed.

The ratio of HDL total core lipid to LDL has been estimated to be ≈1:5 in healthy persons (18), which is similar to the ratios we determined for α-carotene, β-carotene, and lycopene (Table 5). Given the HDL-LDL ratio of 3:1 for lutein and of 2:1 for zeaxanthin, the contents of lutein and zeaxanthin in HDL and LDL would not correlate with their core lipid content.

We suspect that the 3:1 or 2:1 HDL-LDL ratios of lutein and zeaxanthin are more likely to be dependent on the some specific binding or affinity of the xanthophylls to the HDL. This specific affinity of carotenoids to different lipoproteins may then control which tissues the carotenoids are distributed to. For example, those tissues high in LDL receptors, such as the prostate and adrenal glands, would be high in the nonpolar carotenoids β-carotene and lycopene, which is the case (26, 27). Carotenoids carried by HDL containing apolipoprotein E (apo E) are perhaps more efficiently delivered to the central nervous system and retina than from LDL because apo E receptors are abundant in the central nervous system. The preferential uptake of lutein and zeaxanthin from HDL in the retina may be partly explained by the specific binding or affinity of the xanthophylls to the HDL containing apo E, similar to α-tocopherol (28–31).

The crucial role of HDL in the transport of lutein and zeaxanthin is particularly illustrated in the genetic strain of chickens known as WHAM chickens. In this species of chicken there is a mutation of the transporter ABCA1, which results in a very low HDL and an impairment in the transport of lutein and zeaxanthin (32). In particular, the WHAM retina has very low concentrations of lutein and zeaxanthin, ≈5% of the usual concentrations in a normal chicken retina. In the chicken, HDL is the major lipoprotein, so that a deficiency of HDL may significantly impair the transport of lutein and zeaxanthin.

As powerful antioxidants, lutein and zeaxanthin reduce atherosclerotic lesions in animals and reduce the progression of intima-media thickness in human carotid arteries (33). In the
retina, cholesterol accumulation occurs in Bruch’s membrane (34). The similarity of the composition between drusen (oxidized fat, cholesterol esters and protein) associated with AMD and the extracellular deposits associated with atherosclerosis (35) supports the hypothesis that AMD and atherosclerosis may have similar etiologic factors. Perhaps, the protective effects of HDL against cardiovascular disease may be attributed in part to its high content of the antioxidants lutein and zeaxanthin.

The results of this study suggest that patients with AMD may have normal mechanisms to increase plasma concentrations of lutein and zeaxanthin after a diet high in these carotenoids. Furthermore, the transport of these carotenoids was not significantly different between the AMD and control groups in that the lipoprotein HDL was the major transporter, but other lipoproteins, VLDL and LDL, also transported lesser quantities of lutein and zeaxanthin. Because there was no difference in plasma lutein and zeaxanthin concentrations and lipoprotein distribution between the AMD patients and the control group, we suggest that, if there is any defect in the metabolism of lutein and zeaxanthin in patients with AMD, the defect may reside in the uptake of lutein and zeaxanthin from the plasma and transport into the retina. This is a topic that will be the subject of future experiments.

We thank Donna Flavell and Julia Jordan (General Clinical Research Center Biounitritionists) for diet preparation, Carol M Marsh for excellent technical support, and Gary Sexton for statistical help.

WW was responsible for the carotenoid analyses method development, carotenoids and lipid analyses, and data analyses and wrote the first draft of the manuscript. SLC was responsible for diet design and study coordination including supervising the dietary instructions. EJJ was responsible for the initial analysis of carotenoids and provided consultation. MLK was responsible for AMD diagnosis and subject recruitment. SH was responsible for dietary intake data collection and analyses. WEC designed and supervised the study. None of the authors had any personal or financial conflicts of interest.

REFERENCES

Excentral cleavage of β-carotene in vivo in a healthy man\textsuperscript{1–3}

Charlene C Ho, Fabiana F de Moura, Seung-Hyun Kim, and Andrew J Clifford

ABSTRACT

Background: Excentral cleavage of β-carotene to retinoids and apocarotenoids occurs in vitro and in animal models. Whether it occurs in humans is unclear.

Objective: We tested the hypothesis of whether humans can cleave β-carotene excentrally.

Design: A healthy man was given an oral dose of \textit{all-trans} [10,10',11,11'-\textsuperscript{14}C]-β-carotene (1.01 nmol; 100 nCi). Its fate and that of its metabolites were measured in serial plasma samples. Its fate in feces and urine was also measured over time. Selected plasma samples were spiked with reference standards of retinol, β-apo-12'-carotenol, β-apo-8'-carotenal, 13-cis-retinoic acid, all-trans-retinoic acid, β-carotene-5,6-epoxide, \textit{all-trans}-β-carotene, and retinyl palmitate and subjected to reverse-phase HPLC fractionation. The plasma, plasma fractions, urine, and feces were measured for \textsuperscript{14}C with the use of accelerator mass spectrometry.

Results: Sixty-five percent of administered \textsuperscript{14}C was absorbed, and 15.7% was eliminated in urine during the first 21 d after dosing. \textsuperscript{14}C-β-carotene and \textsuperscript{14}C-retinyl palmitate appeared in plasma 0.25 d after the dose. \textsuperscript{14}C-β-carotene and \textsuperscript{14}C-retinol both appeared at 0.5 d only. On day 3 after the dose, 2 large \textsuperscript{14}C peaks appeared in plasma: one matched the retention time of β-apo-8’-carotenal, and the other did not match any of the reference standards used. The delayed appearance of \textsuperscript{14}C-β-apo-8’-carotenal in plasma suggests that the excentral cleavage occurred after the \textsuperscript{14}C-β-apo-8’-carotene was absorbed into the body.

Conclusion: These data suggest that excentral cleavage of ingested β-carotene occurs in vivo in humans. Confirmation of that possibility and further study to identify and characterize additional metabolites are needed. Am J Clin Nutr 2007;85:770–7.

KEY WORDS β-carotene, β-apo-carotenal, humans, vitamin A, \textsuperscript{14}C

INTRODUCTION

Plants and algae synthesize β-carotene that contributes to their color and may confer health benefits as part of diets rich in fruit and vegetables. Approximately 600 carotenoids occur naturally, but only \textlesss than or equal to 60 are found in foods commonly consumed by humans (1). Of these, β-carotene is the best known because, pursuant to its central cleavage to retinal and oxidation to retinol, it can serve as an important source of vitamin A. Excentral cleavage of β-carotene to retinoids and apocarotenoids has been shown in a variety of in vitro systems and animal models. Whether excentral cleavage occurs in vivo in humans and, if so, to what extent is unclear.

When cleaved centrally by β,β-carotene-15,15'-oxygenase [EC 1.13.11.21] (BCO1), β-carotene can yield 2 molecules of retinal (2). When cleaved excentrally, it can lead to 8’-, 10’-, and 12’-apo-carotenals and corresponding alcohols and acids (3). The enzyme β,β-carotene-9’,10’-oxygenase (BCO2) was already identified (4). β-Carotene and its derivatives may have activity (4) aside from vitamin A. These biological activities may include modulating immune response, cellular differentiation, and singlet oxygen quenching (5–7). Therefore, quantification of β-carotene metabolism is paramount to establish its full potential.

Quantifying β-carotene metabolism in the context of fractional absorption, accretion, degradation, and elimination is complex because it can be influenced by the individual consumer, the food matrix, and the consumer-food matrix interactions (8). Previous radioisotope studies estimated intestinal absorption of β-carotene and its conversion to retinyl esters (REs) and to retinal that is subsequently oxidized to retinol (9, 10). However, the conversion of β-carotene by BCO2 to other metabolites, such as β-apo-8’-, 10’-, 12’-, and 14’-carotenals, and retinoic acids (RAs), remains to be determined. Access to accelerator mass spectrometry (AMS) that can measure atto-mole amounts (1 in 10\textsuperscript{−18} parts) of \textsuperscript{14}C (11) enabled us to conduct a feasibility study that tested the hypothesis of whether a healthy man can derive \textsuperscript{14}C-retinoids and \textsuperscript{14}C-apocarotenoids from a true tracer oral dose of \textit{all-trans} [10,10’,11,11’-\textsuperscript{14}C]-β-carotene (1.01 nmol; 543 ng; 100 nCi). The radiation exposure is low, \textapprox \textless 200 nseeirvet.

Therefore, the metabolic fate in a free-living healthy man of \textsuperscript{14}C-β-carotene administered as an oral dose (1.01 nmol; 543 ng; 100 nCi) in a banana milkshake was determined. The fractional absorption, as well as the metabolic fate and elimination of \textsuperscript{14}C-β-carotene, was also measured in the present study.

1 From the Department of Nutrition, University of California, Davis, CA.
2 Supported by the National Center for Research Resources (P41 RR 13461) and the National Institute of Digestive and Kidney Diseases (DK48307), National Institutes of Health. The work was performed under the auspices of the US Department of Energy by the University of California Lawrence Livermore National Laboratory (contract W-7405-Eng-48). Hoffman-La Roche (Basel, Switzerland) donated the \textit{all-trans} [10,10’,11,11’-\textsuperscript{14}C]-β-carotene used in the study.
3 Reprints not available. Address correspondence to AJ Clifford, 3147 Meyer Hall, One Shields Avenue, Department of Nutrition, University of California, Davis, CA 95616-8669. E-mail: ajclifford@ucdavis.edu. Received August 4, 2006. Accepted for publication October 23, 2006.
SUBJECT, MATERIALS, AND METHODS

Chemicals

All chemicals were checked for 14C content by AMS before use. Tributyrin (glycerol tributyrate) was obtained from MP Biomedical (Aurora, OH). All solvent and chemicals used, unless otherwise noted, were obtained from Fisher Scientific (Santa Clara, CA). β-Carotene and all-trans-retinol, retinyl palmitate, all-trans-RA, 13-cis-RA standards were obtained from Sigma Chemicals (St Louis, MO). The β-apo-8-carotenal standard was from Fluka (Buchs, Switzerland), and β-apo-12-carotenal, 9-cis-β-carotene, 13-cis-β-carotene, and β-carotene-5,6-epoxide standards were obtained from Carotenature (Lupsingen, Switzerland). Groceries were purchased at a local supermarket.

Dose preparation

The purity of the all-trans-[10,10′,11,11′-14C]-β-carotene was checked by reverse-phase HPLC (RP-HPLC) as previously described (12). The dose (1.01 nmol; 543 ng; 100 nCi) was suspended in 1 mL ethanol. Specific activity was 98.8 Ci/mmol. Dose formulation was calculated with the use of a liquid scintillation counter (model 1410; Wallac Oy, Turku, Finland). Radioc chemical purity was >99%. A shake that consisted of a whipped mixture of banana, skim milk, and sucrose was prepared and divided between 2 plastic cups. The dose suspended in ethanol was layered over the shake in the first cup. The shake in the second cup was then layered atop that in the first cup as described previously (13). The entire shake was consumed immediately. The first cup was rinsed with water, and the water was then consumed to ensure that the entire dose was ingested. The quantity of dietary β-carotene administered (0.543 µg 14C-β-carotene plus =40 µg β-carotene from the shake) was much smaller than that ingested in a carotene-rich meal.

Subject, diet, and specimen collections

The volunteer was a healthy, nonsmoking man aged 30 y with a body mass index (in kg/m2) of 24.5 who had a normal complete blood count and lipid panel. The subject was instructed to avoid foods with high carotenoid content. A food diary was recorded beginning the week before the study and continuing for 2 wk after dosing, to ensure minimum intake of provitamin A carotenoids and vitamin A. Meals were provided on the day of dose administration to monitor for time and content. Lunch and dinner were served 5.5 and 10 h after dosing; they consisted of a frozen entrée (Amy’s Kitchen Inc, Petaluma, CA), choice of apple or banana, and a chocolate chip cookie (Pepperidge Farm Inc, Norwalk, CT). Meals were selected to deliver 30% fat and minimum vitamin A and carotenoid content.

At 0700 on the day the dose was administered, the (fasting) subject was fitted with an intravenous catheter in a forearm vein, and a blood sample was drawn for baseline values just before dosing. Additional blood samples were collected at 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11, 12, 13, 14, 15, 24, and 36 h after dose delivery. Subsequent blood samples were collected on days 2, 3, 4, 5, 6, 9, 11, 13, 18, 26, 33, 40, 47, 61, 75, 89, 103, 117, 138, and 166 while the subject was in the fasted state. All blood samples were collected in glass K3EDTA evacuated tubes (Vacutainer; BD Diagnostics, Franklin, NJ) and immediately put on ice. A complete collection of feces and urine was taken before dosing to establish baseline values. These collections continued for 14 and 21 d, respectively, after the day of dosing. The subject did not have a history of a serious medical condition or of the use of medications that would interfere with carotenoid metabolism.

Written informed consent was obtained from the subject. The University of California, Davis, and the Lawrence Livermore National Laboratory Institutional Review Boards approved the study, which was conducted according to Good Clinical Practice guidelines and the Declaration of Helsinki, version 1989.

Specimen processing

All specimens were processed under yellow light to prevent degradation of carotenoids and retinoids. Plasma was separated from blood on a fixed-speed bench-top centrifuge (Centriflic model 228; Fisher Scientific, Tustin, CA; 3300 rpm; 1380 × g, 10 min, 25 °C) and stored at −80 °C. Samples were processed under the same conditions as described previously (14) with slight modification. Briefly, fecal samples were collected in polyethylene bags (1-002-59; Fisher Scientific) and homogenized with KOH/2-propanol buffer solution (1:5, wt:vol). Urine samples were collected in amber containers (14-375-115; Fisher Scientific). Representative homogenized samples of feces (50 mL) and urine (15 mL) were stored at −80 °C until they were analyzed further.

Total carbon analysis

Aliquots of urine (75 µL), feces (75 µL), and plasma (25 µL) were lyophilized, wrapped in aluminum foil, and analyzed for total carbon content (15) by using a carbon/nitrogen analyzer (model 1112; Thermo Finnegan, Rodano, Italy). These analyses were conducted at the Division of Agriculture and Natural Resources Laboratory at the University of California, Davis.

Analyte measurements

The approach for isolating β-carotene, retinyl esters (REs), retinol, and RAs involved 3 stages. First, plasma analytes were segregated into neutral (β-carotene, RE, and retinol) and acid-extractable (RA) fractions (12) with modification. Second, neutral and acid-extractable fractions were suspended or comingled in the RP-HPLC mobile phase. Third, the mix was loaded on an RP-HPLC column (ES Industries, West Berlin, NJ) to speciate the analytes in the neutral and acid-extractable fractions with an isotropic mobile phase of acetonitrile:1,4-dioxane:2-propanol:triethylamine (792:148:52:2) with 200 mmol ammonium acetate/L in the alcohol component of the mobile phase. Twenty-second fractions were collected from the RP-HPLC column and processed for AMS analysis as described below.

The neutral fraction, which contained the nonpolar compounds, was extracted from plasma as previously described (13) with a slight modification. Briefly, 200 µL plasma was deproteinated with 400 µL ethanolic KOH (0.25 N). The nonpolar compounds were thrice extracted from the ethanolic KOH with 1 mL hexane each. The extracts were pooled in an amber vial containing 5 µL methanol spiked with 0.25% butylated hydroxytoluene (wt:vol). The solvent was evaporated to dryness under argon, and the residue (nonpolar compounds) was resuspended in 100 µL HPLC mobile phase.

For the acid-extractable fraction, glacial acetic acid (12 µL) was added to the remainder of the deproteinated plasma. Water-saturated ethyl acetate (0.8 mL) was added, the tube was shaken...
and centrifuged (1380 × g for 10 min at 25 °C), and the supernatant fluid containing the acid soluble fraction was transferred to a glass tube (Alltech, Deerfield, IL). Water acidified with 10% glacial acetic acid (300 μL) was added. The tube was shaken and centrifuged (1380 × g for 10 minutes at 25 °C) to separate (float) the ethyl acetate layer that contained the acid-extractable fraction. The ethyl acetate layer was then transferred to an amber screw-top vial containing 5 μL methanol and 0.25% butylated hydroxytoluene (wt:vol). The solvent was evaporated to dryness under a stream of argon. The residue that contained the acid-extractable fraction was resuspended in 100 μL RP-HPLC mobile phase.

The neutrals (described above) were combined or comingled with the acid extractables (described above) in RP-HPLC mobile phase in a total volume of 200 μL. A 20-μL aliquot of the mix was injected onto the HPLC column to separate the various analytes contained in the neutral and acid-extractable fractions of the plasma. RP-HPLC eluant fractions (20 s) were collected and processed for AMS analysis as described below.

An HPLC system (model 1100; Agilent Technology, Santa Clara, CA) equipped with a quaternary pump, an auto sampler, and a photodiode array detector was set up to search for and isolate both the neutral and acid-extractable metabolites of the administered β-carotene. The system was similar to that described previously (16), with modifications. Briefly, the stationary phase consisted of a Spherisorb ODS2 (3 μm; 250 × 4.0 mm) column equipped with titanium frits and a Javelin ODS2 guard column (all: ES Industries). The isocratic mobile phase consisted of acetonitrile:1,4-dioxane:2-propanol:triethylamine (792:148:58:2) with 200 mmol ammonium acetate/L in the alcohol component for 0–25 min, a linear ramp to 100% 2-propanol for 1 min, and a 20-min regeneration of the initial column conditions. The flow rate was 0.8 mL/min, and the column temperature was 35 °C. The HPLC eluent was collected in quartz sample tubes at 20-s intervals for the entire HPLC run.

The system was standardized with 9 reference standards. They included retinol, retinyl palmitate, and retinyl oleate that were monitored at 325 nm; 13-cis-RA and all-trans-RA that were monitored at 351 nm; and β-apo-12’,13’-carotenal, β-apo-8’,9’-carotenal, β-carotene-5,6-epoxide, and all-trans-β-carotene that were monitored at 450 nm. The parent compound, 14C-β-carotene, and its 14C-metabolites 14C-retinol, 14C-retinyl palmitate, and 14C-β-apo-8’,9’-carotenal were separated with the use of the above standardized HPLC system.

AMS analysis

The 20-s RP-HPLC eluent fractions described above were processed as previously described (13), except that a 50-μL aliquot of a solution of tributyrin in methanol (40 mg/mL) was used to add exactly 1.2 mg C to each 20-s RP-HPLC fraction, which was then dried under vacuum to remove the mobile phase and methanol. An aliquot of urine was prepared for 14C analysis by diluting 100 μL of the sample with 900 μL HPLC-grade water. Aliquots of diluted urine (100 μL) and feces (75 μL) were placed in quartz tubes and processed as described for the 20-s RP-HPLC fractions. Carbon in the samples was converted to graphite (17), and the ratio of 14C to totalC was measured at the Center for Accelerator Mass Spectrometry, Lawrence Livermore National Laboratory, Livermore, CA.

Statistical analysis

The 14C content in the whole plasma, urine, and feces was plotted as a function of time since dosing, whereas the 14C content in the HPLC fractions was plotted as a function of retention time. Plotting was performed with ORIGINLAB software (version 7.5; Microcal, Northampton, MA).

RESULTS

A schematic presentation of some possible cleavage pathways and metabolites of all-trans-β-carotene is shown in Figure 1. Central cleavage at the 15,15’ position is the well-established pathway that leads to vitamin A. Cleavage at the 3’–4’, 5’–6’, 7’–8’, and 9’–10’ positions, were it to occur in humans, would lead to β-apo-carotenals that may be converted to β-apo-carotenols, β-apo-carotenyl esters, β-apo-carotenonic acids, β-apo-carotenoyl esters, and the ring-oxidized metabolites (oxo-, hydroxy-, and epoxy- forms).

14C in neat plasma

The profile of 14C in neat plasma by time since dose included 4 peaks, as shown in Figure 2. Peak A appeared at 0.15 d after dosing and represented ≈3% of the dose (100 nCi dose had 1.6 × 106 amol 14C/mg carbon). Peak B appeared at 0.25 d after dosing and represented ≈3.6% of the dose. Peak C appeared at 0.5 d and represented ≈2.8% of the dose. Peak D appeared on the third day after dosing; it was poorly resolved.

HPLC separation of reference standards

The separation of the 9 reference standards is shown in Figure 3A. Respective retention times for the retinoids were 3.8, 6.5, 7.5, 18.5, and 21.5 min for retinol, 13-cis-RA, all-trans RA, retinyl palmitate, and retinyl stearate. Respective retention times for the carotenoids were 4.6, 5.5, 10.5, and 15.5 min for β-apo-12’,13’-carotenal, β-apo-8’,9’-carotenal, β-carotene-5,6-epoxide, and all-trans-β-carotene.

HPLC separation of 14C analytes in plasma

The 14C analytes in plasma 0.25 d after administration of 14C-β-carotene are shown in Figure 3B. At this time since dosing, the 14C peaks matched the retention times for β-carotene, retinyl palmitate, and retinyl stearate. The 14C analytes in plasma 0.5 d after administration of 14C-β-carotene are shown in Figure 3C. At this time since dosing, the 14C peaks matched the retention times for retinol and β-carotene. The 14C analytes in plasma 3 d after administration of 14C-β-carotene are shown in Figure 3D. At this time since dosing, the first 14C peak matched the retention times for β-apo-8’,9’-carotenal, whereas the second 14C peak that eluted immediately after 17 min did not match that of any of our reference standards. More reference standards are needed to identify this 14C metabolite. The possibility that the first peak corresponds to β-apo-10’-carotenal (rather than to β-apo-8’,9’-carotenal) cannot be ruled out, for lack of that reference standard.

Plasma 14C-RE and 14C-retinol by time since dosing

The profiles of 14C-RE and 14C-retinol in plasma at all sampling times since dosing are shown in Figure 4. The rise of a 14C-RE peak at 0.15 d after dosing represents delivery of the 14C retinoid from enterocytes to chylomicra, and the descent represents the presence of 14C-RE in chylomicron remnants as they are
taken up by the liver. The rise of $^{14}$C-retinol that began shortly after 0.2 d after dosing represents the handing off of $^{14}$C-RE to $^{14}$C-retinol bound to retinol-binding protein for secretion into plasma.

**Plasma $^{14}$C-carotenoids by time since dosing**

The plasma profiles of $^{14}$C-$\beta$-carotene and $^{14}$C-$\beta$-apo-8'-carotene are shown in Figure 5. The $^{14}$C-$\beta$-carotene appeared as 2 broad peaks. The most interesting feature of the $^{14}$C-apo-8'-carotene peak is that, although it was not present at 0.5 d, it was clearly present on the third day after dosing.

**Elimination of $^{14}$C in feces**

Elimination of the $^{14}$C label in feces appears in Figure 6. The first collection of feces after dosing accounted for $\approx 35\%$ of the administered $^{14}$C, so the fractional absorption (apparent digestibility) was $\approx 0.65$ ($1.00 - 0.35$). Subsequent collections during the next 13 d accounted for an additional $16\%$ of the administered $^{14}$C, so the fractional daily elimination (metabolic fecal loss resulting from biliary excretion of metabolites such as retinoyl $\beta$-glucoronide) of the label was $\approx 0.0123$ ($0.16/13$). The cumulative elimination of $^{14}$C in feces was $51\%$ of the administered $^{14}$C during the 2-wk period since dosing.
FIGURE 2. Plasma profile of radioactivity after an oral tracer dose of $^{14}$C-$\beta$-carotene (1.01 nmol, 543 ng, 100 nCi) expressed in modern as a function of days after dose on a log scale for 166 d. Total $^{14}$C label (○) in plasma was assessed by accelerator mass spectrometry. The $^{14}$C peaks are denoted by the letters A, B, C, and D. One modern = 97.94 amol $^{14}$C/mg carbon; dose = 100 nCi = $1.6 \times 10^9$ amol $^{14}$C/mg carbon. $^{14}$C metabolites in peaks B, C, and D are shown in Figure 3.

FIGURE 3. RA, retinoic acid. A: The chromatogram of retinoid and carotenoid reference standards on an isocratic HPLC system by retention time. The retinoid standards were measured at 325 nm; the carotenoid standards were measured at 450 nm. $^{14}$C metabolites in plasma from orally administered $^{14}$C-$\beta$-carotene are expressed in modern by accelerator mass spectrometry. B: $^{14}$C plasma profile at 0.25 d after dose; C: $^{14}$C plasma profile at 0.5 d after dose; D: $^{14}$C plasma profile at 3 d after dose. Plasma samples were separated by HPLC and collected at 20-s intervals for analysis by accelerator mass spectrometry. Modern = 97.94 amol $^{14}$C/mg C.
Elimination of $^{14}$C in urine

Elimination of $^{14}$C in urine appears in Figure 7. In the cumulative collections, $\approx 15.7\%$ of the administered $^{14}$C was eliminated during the first 21 d since dosing. An interesting feature of the elimination per collection line is the peak that appears in the day 4 and day 5 collections. It may represent the elimination of either the $^{14}$C-β-apo-8′-carotenal or the unknown, or both, which were discovered in plasma 3 d after dosing (Figure 3D). The first urine collection (0–12 h since dosing) accounted for 1.1% of the administered dose. The day 4 and day 5 collections accounted for 2.3% and 1.7% of the administered dose, respectively, and each subsequent collection accounted for $\approx 0.2\%$.

DISCUSSION

A quantitative understanding of β-carotene metabolism is of considerable interest because it is cleaved by a sequence-related family of retinal- and apo-carotenal–forming carotenoid oxygenases to retinoids and apo-carotenoids (Figure 1) that perform essential biological functions (18, 19). The best-known of the oxygenases in humans is the BCO1 that cleaves β-carotene to vitamin A (2, 20, 21). A related cleavage enzyme (BCO2) that cleaves β-carotene to β-apo-carotenals was also identified and characterized (4). BCO2 is widely distributed among human tissue (22). Another related oxygenase (RPE65) cleaves all-trans-RE to 11-cis-retinol that is isomerized to 11-cis-retinal through the visual cycle (23). Therefore, central and excentral β-carotene cleavage enzymes occur in humans, but the excentral cleavage metabolites have not been reported. Therefore, we tested whether excentral cleavage was demonstrable in vivo in humans.

We administered a small oral tracer dose of $^{14}$C-β-carotene. The mass of the dose was 1.01 nmol; the typical dietary intake in the United States is 7 μmol/d. The small dose and carefully monitored diet ensured steady state conditions with respect to carotenoid metabolism. We quantified the elimination of the dose in feces and urine for 14 and 21 d, respectively, and we searched plasma for $^{14}$C-apo-carotenals with the use of HPLC and AMS.

We found that the percentage of absorption was $\approx 65\%$ of the administered dose, metabolic fecal loss was $\approx 1\%$ of the administered dose per day (Figure 6), and urine loss was $\approx 0.75\%$ of the...
administered dose per day (Figure 7). These findings were all consistent with prior estimates (24–26), which suggests that the subject was normal with respect to β-carotene metabolism.

In searching the blood, we found 14C-β-carotene and 14C-REs at 0.25 d and 14C-retinol and 14C-β-carotene at 0.5 d as we had expected (Figures 3, 4, and 5). Our finding of ≥2 isotope-labeled metabolites from administered 14C-β-carotene at 0.25 and 0.5 d confirms previous observations of 2 peaks (12, 13, 27). Finally, we found 14C-β-apo-8'-carotene, 14C-β-carotene, and a 14C peak that could be a 14C-β-apo-8'-caroteryl ester or a 14C-β-apo-8'-carotenoyl carbonate. Our finding of a first peak of 14C-β-apo-8'-carotene is consistent with results of a prior study that incubated β-carotene with tissue homogenates and found β-apo-8'-, 10'-, and 12'-carotenals, retinal, and RA (28). Our finding of a second 14C peak, tentatively identified as a 14C-β-apo-8'-carotenoyl ester, or 14C-β-apo-8'-carotenoyl carbonate, fits nicely with a prior study that incubated retinal, β-carotene, β-apo-8'-carotene, or β-apo-12'-carotene with a homogenate of human intestinal mucosa and found RA (29). Our finding of a second 14C peak fits nicely with a prior study showing that orally administered β-apo-8'-carotene was extensively and rapidly converted to its corresponding acid, alcohol, and fatty acyl ester (30). Finally, our finding of 14C-β-carotene and 14C-β-apo-8'-carotene in plasma fits nicely with our previous evidence of 2 kinetically distinct pools of carotenoid (27).

At 3 d after dosing, 90% of the total 14C label (present in neat plasma) was recovered in the HPLC fraction (Figure 3D). Of the 90% recovered, 20% appeared with β-apo-8'-carotenoid reference standard, 41% coeluted with a second large peak at 17 min (its chemical identity is unknown at this time), and the remaining 29% consisted of small peaks of 14C with retention times of 6–15 min (Figure 3D), which could represent additional apocarotenoid metabolites. Cleavage activity by BCO2 is shown to correlate, alcohol, and fatty acyl ester (30). Finally, our finding of a second 14C peak fits nicely with our previous evidence of 2 kinetically distinct pools of carotenoid (27).

In animals, carotene cleavage dioxygenases (CCDs) include BCO1 (2), BCO2 (4), and RPE65 (23). However, our feasibility study tentatively identified the formation of β-apo-8'-carotenol and perhaps β-apo-8'-caroteryl ester or β-apo-8'-carotenoyl carbonate by exscentral cleavage of β-carotene in a normal human. This is a new finding that requires confirmation and, if confirmed, further investigation in additional subjects.

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AJC designed the study and wrote the manuscript, and CCH, FFM, and SHK were responsible for sample preparation and analyses. None of the authors had a personal or financial conflict of interest.

REFERENCES


22. Lindqvist A, He Y-G, Andersson S. Cell-type-specific expression of...


Iron treatment normalizes cognitive functioning in young women\textsuperscript{1–4}

Laura E Murray-Kolb and John L Beard

ABSTRACT

Background: Evidence suggests that brain iron deficiency at any time in life may disrupt metabolic processes and subsequently change cognitive and behavioral functioning. Women of reproductive age are among those most vulnerable to iron deficiency and may be at high risk for cognitive alterations due to iron deficiency.

Objective: We aimed to examine the relation between iron status and cognitive abilities in young women.

Design: A blinded, placebo-controlled, stratified intervention study was conducted in women aged 18–35 y of varied iron status who were randomly assigned to receive iron supplements or a placebo. Cognition was assessed by using 8 cognitive performance tasks (from Detterman’s Cognitive Abilities Test) at baseline \((n = 149)\) and after 16 wk of treatment \((n = 115)\).

Results: At baseline, the iron-sufficient women \((n = 42)\) performed better on cognitive tasks \((P = 0.011)\) and completed them faster \((P = 0.038)\) than did the women with iron deficiency anemia \((n = 34)\). Factors representing performance accuracy and the time needed to complete the tasks by the iron-deficient but nonanemic women \((n = 73)\) were intermediate between the 2 extremes of iron status. After treatment, a significant improvement in serum ferritin was associated with a 5–7-fold improvement in cognitive performance, whereas a significant improvement in hemoglobin was related to improved speed in completing the cognitive tasks.

Conclusions: Iron status is a significant factor in cognitive performance in women of reproductive age. Severity of anemia primarily affects processing speed, and severity of iron deficiency affects accuracy of cognitive function over a broad range of tasks. Thus, the effects of iron deficiency on cognition are not limited to the developing brain.

KEY WORDS
Iron, women, cognition, attention, memory, learning, ferritin, hemoglobin

INTRODUCTION

Despite advances in the reduction of a number of nutrient deficiencies worldwide, iron deficiency (ID) remains the most prevalent single nutrient deficiency, and it affects those in both developing and developed countries. The World Health Organization (WHO) estimates that, worldwide, 2 billion people are anemic and twice as many are iron deficient \((1)\). Because of their greater physiologic requirements, combined with increased losses and poor dietary intake, those at highest risk of developing ID and iron deficiency anemia (IDA) are infants, children, and women of reproductive age.

Nonhematologic manifestations of ID include reduced physical endurance, an impaired immune response, difficulty in regulating temperature, changes in energy metabolism, decreased cognitive performance, and behavioral disturbances \((2–4)\). Over the past 30 y, a large effort has focused on understanding the relation between ID and development or behavior in infants and young children \((4–8)\). As a result, we have strong evidence that IDA is associated with poorer performance on developmental ratings in infants and with lower scores on cognitive function tests and educational achievement tests in children. In adolescents, ID has been shown to impair cognitive abilities even in the absence of overt anemia \((9)\).

Whereas data on the relation between iron status and cognition is mounting in both infants and children, a gap exists in our understanding of this same relation in adults. The large number of studies conducted in infants has led many to assume that ID disrupts brain functioning only during development \((10)\). However, new evidence from animal models and in humans with restless leg syndrome (RLS) suggests that brain ID at any time in life is likely to disrupt metabolic processes and to be followed by changes in cognitive and behavioral functioning \((4)\).

Reports have been published of cognitive improvement in adult renal dialysis patients who are receiving both erythropoietin and iron supplementation as part of treatment protocols \((11–13)\), as well as in elderly whose iron nutritional status has improved \((14)\). Recently, we reported a relation between iron status and cognitive abilities in poor South African mothers during the first postpartum year \((15)\). Whereas these studies all point to a relation between iron status and cognition, the numerous confounding variables in each of them make the findings difficult to assess. Therefore, we undertook a more thorough investigation of the relation between iron status and cognition in women of reproductive age by conducting a blinded, placebo-controlled, intervention study. The overall aim of the study was to examine

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the effects of ID and IDA on cognitive and emotional performance in young women. Here we report the results of the cognitive tasks. The primary outcomes of the study were the relation between iron status and cognitive task performance and that between changes in iron status and changes in cognition. We also wanted to examine whether different facets of cognition were differentially affected by iron status or a change in iron status. Given estimates that up to 50% of women in the world are iron deficient, a documented relation between iron status and cognitive abilities could provide a basis for interventions that would be relevant to the psychological functioning of a significant proportion of the world’s population.

SUBJECTS AND METHODS

Subjects
This study was conducted on the University Park campus of The Pennsylvania State University in State College, PA between the fall of 1999 and the fall of 2002. Women aged 18–35 y were recruited via flyers as well as advertisements in the local newspaper. Interested women reported to the General Clinical Research Center (GCRC) for screening, which consisted of a venous blood draw and the completion of a health history questionnaire. The criteria for participation in the study included the following: female between 18 and 35 y of age, free from any chronic illness or serious health problems and where English is the primary language spoken in the home.

Written informed consent was obtained from each subject. All procedures used in this study were reviewed and approved by the Institutional Review Board at The Pennsylvania State University and were in accordance with the Helsinki Declaration of 1975 as revised in 1983.

Methods
The blood samples were used for the measurement of a complete blood count (including hemoglobin and hematocrit), serum ferritin (sFt) (Diagnostic Products Corporation, Los Angeles, CA), serum transferrin receptor (sTIR) (Ramco, Houston, TX), and plasma iron and total iron–binding capacity (TIBC) by standard methods (16). Transferrin saturation was then calculated as and plasma iron and total iron–binding capacity (TIBC) by standard methods (16). Transferrin saturation was then calculated as

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\text{Transferrin saturation} = \frac{\text{Plasma iron}}{\text{TIBC}} \times 100
\]

C-reactive protein was also measured.

Women were classified as iron sufficient (control group; CN), nonanemic but with iron deficiency (ID group), or with iron deficiency anemia (IDA group). IDA was defined as a hemoglobin concentration between 105 and 119 g/L and ≥2 abnormal iron status values. ID was defined as above, except that hemoglobin concentrations had to be ≥120 g/L. Women with hemoglobin < 105 g/L were excluded from the study and referred to a physician for treatment. Once the women were stratified into the 3 iron status groups, they were randomly assigned (stratified randomization performed by using random permuted blocks) to receive either a slow-release iron supplement (160 mg ferrous sulfate containing 60 mg elemental iron) or a placebo. Only one of the researchers knew the allocation of the subjects, and the researchers responsible for testing the women were unaware of treatment allocation. The researcher who knew the treatment allocation was responsible for preparing the bottles of supplements to be given to the subjects. The bottles were labeled only with the subject’s identification number and with the instruction to “take 1/d for the next 16 wk” (the women were also told to keep the pills away from children). A 4-mo intervention was chosen for 2 reasons. First, we wanted to ensure that the women given iron supplements would be truly iron replete at the end of the study. Second, evidence exists in animals that liver iron concentrations are replenished at a faster rate than are brain iron concentrations (18). Therefore, whereas studies have reported increases in hemoglobin and hematocrit concentrations after subjects have consumed iron supplements for 8–12 wk, we wanted to allow more time for brain iron concentrations to be replenished; thus, we chose a period of 16 wk. Our allocation of subjects to consume either iron or placebo resulted in 6 treatment groups: CN group taking placebo (CNPL), CN group taking iron (CNFe), ID group taking placebo (IDPL), ID group taking iron (IDFe), IDA group taking placebo (IDAPL), and IDA group women taking iron (IDAFe). The subjects were asked to refrain from taking any vitamin or mineral supplements during the trial.

Cognitive and emotional testing was conducted both at baseline and after 16 wk of treatment. Each woman first completed a questionnaire that obtained information about university grade-point average, level of physical activity, demographic variables (socioeconomic status (SES) was determined from the mother’s and father’s occupations and education level with the use of the Hollingshead 2 factor index; 19], oral contraceptive use, and menstrual cycle information. The subjects then completed the Shipley Institute of Living Scale (20), a self-administered test consisting of 2 subtests: a vocabulary subtest and an abstraction subtest. Scores from this scale were used to estimate intelligence quotient (IQ) for each woman through the use of a continuously adjusted age norms method (21).

Next, each woman completed 8 self-administered and automated computerized tasks of basic cognition using the Cognitive Abilities Test (CAT; 22). One of the advantages of the CAT is that it has been subject to the same psychometric rigors commonly used to develop intelligence tests. High reliabilities have been found for each of the tasks present in the CAT (23). These tasks were developed to measure the “modal model” of information processing that offers the opportunity to test specific aspects of cognition. This model is composed of 3 memory stores (very-short-term memory, short-term memory, and long-term memory), which are served by a stimulus encoding mechanism for input, a retrieval mechanism for output, and an output mechanism that executes responses. It also contains an executive functioning mechanism that oversees movement of information through the system.

Instructions appeared before each test and consisted of 3 parts—written instructions, clarification by the tester, and practice trials. The tests measure 3 domains—attention, memory, and learning—and correspond to levels of complexity as follows: attention: tachistoscopic threshold, reaction time, and stimulus discrimination; memory: probe recall, Sternberg memory search, and recognition memory; learning: and learning and progressive matrices.

The reaction time task includes both simple and choice reaction times in which several measures of speed and accuracy are obtained. The stimulus discrimination task is a modified match-to-sample test that yields measures of stimulus encoding and search processes. To establish a threshold, the tachistoscopic
threshold task measures the minimum amount of time needed for a subject to decide whether 2 stimuli are the same or different. These 3 measures of attention probe very-short-term memory as well as encoding and output mechanisms of information processing.

The probe recall task yields measures of memory, accuracy, and speed. The recognition memory task is a forced-choice recognition test used to measure memory, speed, and accuracy. The Sternberg memory search task is used to measure the amount of time it takes to search easy stimulus sets of various sizes. This test yields measures of memory, speed, and accuracy for 4 different set sizes. These 3 measures of memory probe short-term memory and the retrieval and executive functioning mechanisms of information processing.

The learning task requires subjects to encode increasingly larger sets of stimuli to measure learning rate, whereas the progressive matrices task is modeled after the progressive matrices type of intelligence tests and is the most complex of the tasks. These 2 measures of learning probe long-term memory and analytic reasoning.

After baseline testing, each subject was given either a placebo or a slow-release iron supplement (160 mg ferrous sulfate containing 60 mg elemental iron) and instructed to take 1 dose/d for the next 4 mo. After 16 wk, the women returned for a venous blood draw and repeated cognitive testing. Those women who were found to be anemic at the end of the study were provided with 30 days’ worth of supplements and referred to their physician.

**Statistical analysis**

All data were analyzed with SAS for WINDOWS software (version 8e; SAS Institute, Cary, NC). Log transformation of ferritin as well as transferrin receptor variables was required for normalization. Factor analysis (principal axis with varimax rotation) was carried out for the cognitive and hematologic variables to reduce the number of variables and the probability of a type 1 error. The factors were then used for the analyses. Test scores were standardized by calculating z scores to facilitate the comparison of scores across the domains. Therefore, the scores have an SD of 1. Differences between groups (CN, ID, and IDA) at baseline were examined by using analysis of covariance (ANCOVA) with IQ as a covariate and Tukey’s test as the post hoc test.

To examine change in the cognitive variables over time, repeated-measures analyses were employed after adjustment for IQ and baseline value on any particular task. Analyses were run with women classified as ferritin responders or nonresponders and hemoglobin responders or nonresponders. This step was taken because the current study was designed to determine the relation between changes in iron status and changes in cognition. Therefore, in keeping with our longitudinal hypothesis, which stated that an intervention that normalized the iron status of the young women would normalize their cognitive scores, data will be presented as comparing responders and nonresponders with respect to iron treatment, regardless of original group assignment. If we were to ignore our hypothesis and simply keep the group assignment as originally determined at baseline, a large potential would exist for erroneous conclusions because of the inclusion of women with no change in iron status in a group in which change may have been expected (or vice versa). Classifying the women as responder and nonresponders was done on an individual basis after determining whether the woman experienced a change in ferritin or hemoglobin greater or less than the known biological day-to-day variation (24) for each of these iron status variables. The analyses were then run by using repeated-measures ANCOVA with Tukey’s test as the post hoc test.

**RESULTS**

The progress through the phases of this trial, which was carried out over a 3-y period, can be found in Figure 1. Of the 398 women who were screened, 152 were deemed eligible, stratified according to iron status, and randomly assigned to treatment. At follow-up, 113 women returned to complete the study. There were no differences between the groups at baseline with respect to mean age (21 ± 3 y), ethnic distribution, SES, birth control use, reported physical activity level, oral contraceptive use, GPA, or menstrual cycle characteristics (data not shown). Further exploration found no significant relation between these variables and iron status or cognition. Within each group, no significant hematologic differences were found between the women randomly assigned to placebo and those randomly assigned to iron at baseline.

Hematologic measurements are given in Table 1. As per the design of the study, at baseline, the iron status of the groups was significantly different. None of the women was found to have any indication of inflammation, as evidenced by negative results on the CRP test. At endpoint, those groups receiving iron had significantly improved their iron status. Significant increases occurred in the IDFe and IDAfFe groups for ferritin (P < 0.001 and P < 0.01, respectively) and body iron (P < 0.0001 and P < 0.01, respectively). The IDAfFe group also experienced a significant increase in hemoglobin (P < 0.0001), hematocrit (P < 0.001), and transferrin saturation (P = 0.018) and a nonsignificant decrease in transferrin receptor (TfR) concentrations. Groups consuming the placebo also experienced some hematologic changes over time. The CNPL group had a significant decrease in transferrin saturation (TSAT)(P = 0.010). However, for the IDPL and IDAPL groups, there was a regression to the mean with respect to ferritin (IDPL) and to hemoglobin and hematocrit concentrations (IDAPL), which rendered these groups no different at endpoint from their iron-receiving counterparts with respect to these variables. Of the women with the lowest hemoglobin values at baseline (<110 g/L), 67% experienced an increase in hemoglobin > 10 g/L, whereas only 10% of the women whose hemoglobin values were >120 g/L at baseline did so (Figure 2).

The factor analysis carried out on the hematologic variables found 4 factors, which we termed storage, transport, preanemia, and anemia (Table 2). Factor analysis of the cognitive variables within each cognitive domain (ie, attention, memory, and learning) found 2 factors for each domain tested. The factors were termed performance factor and time factor (Table 3). Factor analysis of all cognitive variables together found 2 overall factors, which were termed performance and time (Table 3). Data are represented by each of these factors (performance and time) as well as by a composite score that consists of the performance factor minus the time factor.

**Cross-sectional baseline comparisons**

Results of the overall scores (all cognitive domains considered together) are shown in Figure 3. The composite score shows that women in the CN group scored the highest, whereas women in the IDA group scored the lowest. Women in the ID group scored between women in the CN and IDA groups. Differences were
found to be significant between the CN and IDA groups and between the ID and IDA groups ($P < 0.001$ for both). Scores on the performance factor followed this same pattern (CN > ID > IDA), and the differences were significant between the CN and IDA groups ($P = 0.011$) and between the ID and IDA groups ($P = 0.007$). The pattern for the score on the time factor was exactly the opposite (IDA > ID > CN), and the differences between the CN and IDA groups ($P = 0.038$) and the ID and IDA groups ($P = 0.036$) were significant. These scores indicate that not only was the CN group able to perform better on the cognitive tasks, but those women also were able to do so in a shorter amount of time.

Categorizing the women into the CN, ID, and IDA groups uses data that is continuous (iron status data) and places it into discrete categories (through the use of accepted cutoffs), thereby diminishing the power of the continuous data. To utilize the full power of our measures, data were sorted according to each hematologic factor (ie, storage, transport, preanemia, and anemia) and then divided into quintiles. ANCOVAs were then run on the data with the intention of specifically examining the extremes of the distribution. Analyses comparing the upper and lower quintiles for the storage factor found significantly better performance by those in the upper quintile (0.12 compared with −0.31; $P = 0.030$) but no difference in the time necessary to complete the tasks. The opposite was found when comparing the upper and lower quintiles for the anemia factor. That is, women in the highest quintile required significantly less time to complete the tasks than did those in the lower quintiles (−0.11 and 0.18, respectively; $P = 0.020$), but performance levels did not differ significantly between the quintiles.

To obtain a better understanding of exactly where the cognitive deficits lie, we then analyzed the cognitive tasks by cognitive domain (ie, attention, memory, and learning). Results from these analyses are shown in Figure 4. With respect to the attention domain (Figure 4A), the composite score did not differ between the CN and ID groups but was significantly lower in the IDA group than in the CN group ($P = 0.008$) or the ID group ($P = 0.003$). Performance in the attention domain did not differ between the CN and ID women but was significantly better than that of the IDA women ($P = 0.047$ and 0.008, respectively). However, women in the CN group completed the tasks quicker than did women in either the ID or IDA groups. Whereas these differences did not reach statistical significance, the difference between the CN and IDA groups trended toward significance ($P = 0.064$).

Composite scores on the memory domain (Figure 4B) show the CN and ID groups scoring equally well and significantly better than the IDA group ($P < 0.001$ for both). The performance
The 3-way interaction (time × factor on the memory domain shows this same pattern, but the time factor shows the opposite. Differences with respect to the performance factor were significant between CN and IDA groups (P = 0.002) and between ID and IDA groups (P < 0.001).

The z scores for the time factor did not differ significantly between the CN and IDA groups (P = 0.159) but did differ significantly between the ID and IDA groups (P = 0.038).

Finally, the composite scores on the learning domain (Figure 4C) follow the pattern CN > ID > IDA with a significant difference between the CN and IDA groups (P = 0.013) and the ID and IDA groups (P = 0.042). The difference between the groups on

**TABLE 1**
Hematologic variables in subjects at baseline (n = 149) and endpoint (n = 113)1

<table>
<thead>
<tr>
<th>Hematologic variables</th>
<th>Control group</th>
<th>Iron deficiency group</th>
<th>Iron deficiency anemia group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CNPL (n = 21, 16)1</td>
<td>IDPL (n = 37, 28)</td>
<td>IDAPL (n = 15, 13)</td>
</tr>
<tr>
<td></td>
<td>CNFe (n = 21, 14)</td>
<td>IDFe (n = 36, 25)</td>
<td>IDAFe (n = 19, 17)</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>139 ± 9a</td>
<td>133 ± 6b</td>
<td>114 ± 5c</td>
</tr>
<tr>
<td>Endpoint</td>
<td>142 ± 11</td>
<td>132 ± 10</td>
<td>121 ± 9d</td>
</tr>
<tr>
<td>Hct (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42 ± 3a</td>
<td>41 ± 2b</td>
<td>35 ± 1c</td>
</tr>
<tr>
<td>Endpoint</td>
<td>44 ± 5</td>
<td>41 ± 4</td>
<td>37 ± 3d</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>90.4 ± 2.2a</td>
<td>88.2 ± 3.4a</td>
<td>79.7 ± 8.0e</td>
</tr>
<tr>
<td>Endpoint</td>
<td>90.2 ± 3.3</td>
<td>87.5 ± 4.1</td>
<td>81.8 ± 6.4e</td>
</tr>
<tr>
<td>RDW (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>12.7 ± 0.5a</td>
<td>13.4 ± 1.0b</td>
<td>14.4 ± 1.1e</td>
</tr>
<tr>
<td>Endpoint</td>
<td>13.0 ± 0.7</td>
<td>14.0 ± 1.0</td>
<td>15.0 ± 1.0</td>
</tr>
<tr>
<td>sFt (μg/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>145 ± 5.04a</td>
<td>6.5 ± 0.8e</td>
<td>9.1 ± 2.7d</td>
</tr>
<tr>
<td>Endpoint</td>
<td>142 ± 2.7a</td>
<td>6.4 ± 2.6</td>
<td>7.9 ± 1.3</td>
</tr>
<tr>
<td>sTfSat (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>33 ± 11a</td>
<td>24 ± 12b</td>
<td>19 ± 9b</td>
</tr>
<tr>
<td>Endpoint</td>
<td>23 ± 9a, b</td>
<td>24 ± 14b</td>
<td>16 ± 7b</td>
</tr>
<tr>
<td>Body iron (mg/kg)1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>6.5 ± 1.5a</td>
<td>-0.4 ± 2.0b</td>
<td>-4.0 ± 3.6c</td>
</tr>
<tr>
<td>Endpoint</td>
<td>5.8 ± 2.2a, c</td>
<td>0.6 ± 3.9b</td>
<td>-1.9 ± 3.2b</td>
</tr>
</tbody>
</table>

1 All values are x ± SD. Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; RDW, red blood cell distribution width; sFt, serum ferritin; sTfR, serum transferrin receptor; TfSat, transferrin saturation; CNPL, control group receiving placebo; CNFe, control group receiving iron; IDPL, iron deficiency group receiving placebo; IDFe, iron deficiency group receiving iron; IDAPL, iron deficiency anemia group receiving placebo; IDAFe, iron deficiency anemia group receiving iron. Values within a row with different superscript letters are significantly different, P < 0.05. ANOVA was used to compare groups at a given time point, and repeated-measures analyses were used after adjustment for baseline values to analyze change over time. Tukey’s test was used to compare the groups at baseline and endpoint. The 3-way interaction (time × factor × treatment) was significant (P < 0.05) for all variables except MCV and RDW. Two-way interaction (group × pills) was significant (P < 0.01) for sFt, TfSat, and body iron; 2-way interaction (group × time) was significant (P < 0.05) for all variables except MCV and RDW.
2 n at baseline, endpoint (all such).
3 Significantly different from baseline, P < 0.05.
4 Calculated by method of Cook et al (17).

**FIGURE 2.** Change in hemoglobin concentration as a function of initial hemoglobin status (P < 0.0001, chi-square test). □, Hemoglobin change >10 g/L; ▪, hemoglobin change 5–10 g/L; △, hemoglobin change <5 g/L.
The performance or time factor for the learning domain was not significant.

The use of the full power of our continuous iron status data by sorting according to our hematologic factors also showed a difference in performance that trended toward significance (for the attention and learning domains) when the upper and lower quintiles for storage factor were analyzed (attention: 0.01 compared with 0.26; \( P = 0.091 \); memory: 0.03 compared with 0.25; \( P = 0.130 \); learning: 0.31 compared with 0.43; \( P = 0.061 \)), whereas no difference was found in the time necessary to complete the tasks for the attention and learning domains. However, the amount of time needed to complete the memory tasks differed significantly between the upper and lower quintiles (−0.09 compared with 0.22; \( P = 0.031 \)). In contrast, when the data were compared between the upper and lower quintiles for the anemia factor, the women in the highest quintile completed the tasks in the attention domain significantly faster than did the women in the lowest quintile (−0.11 compared with 0.19, respectively; \( P = 0.035 \)) but no differences were found for the memory and learning domains (memory: −0.12 compared with 0.09, respectively, \( P = 0.151 \); learning: −0.10 compared with 0.19, respectively, \( P = 0.164 \)). In contrast, performance on the attention, memory, and learning domains did not differ significantly between the upper and lower quintiles for the anemia factor.

### TABLE 3

<table>
<thead>
<tr>
<th>Component of cognitive factors</th>
<th>Performance factor</th>
<th>Time factor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Attention</strong></td>
<td>RT, SD, TT: no. incorrect</td>
<td>RT, SD, TT: trial time</td>
</tr>
<tr>
<td></td>
<td>TT: no. attempted trials</td>
<td>RT, SD, TT: decision time</td>
</tr>
<tr>
<td><strong>Memory</strong></td>
<td>PR, RC: % correct</td>
<td>PR, RC, ST: trial time</td>
</tr>
<tr>
<td></td>
<td>ST: no. incorrect</td>
<td>RC, ST: decision time</td>
</tr>
<tr>
<td><strong>Learning</strong></td>
<td>LR: no. attempted trials</td>
<td>LR: trial time</td>
</tr>
<tr>
<td></td>
<td>LR: % correct</td>
<td>LR, PM: reaction time</td>
</tr>
<tr>
<td><strong>All domains</strong></td>
<td>All of the variables listed above</td>
<td>All of the variables listed above</td>
</tr>
</tbody>
</table>

1 RT, reaction time task; SD, stimulus discrimination task; TT, tachistoscopic threshold task; PR, probed recall task; RC, recognition memory task; ST, Sternberg memory search task; LR, learning task; PM, progressive matrices task. Factor analysis (principal axis with varimax rotation) was used for statistical analysis.

2 For those tasks that measured “negative” performance, the absolute values of the scores were used; therefore, a higher score on the performance factor is always indicative of better performance.
The hemoglobin responders did not carry over into an improvement. This decrease in the time necessary to complete the tasks for completion times at the end of the study than at the beginning (Figure 6). The women who showed a significant change in hemoglobin (hemoglobin responders, n = 66) and nonresponders (■, n = 47) with respect to ferritin change. Significantly different from baseline: **P < 0.01, †P < 0.05. Analyses conducted with repeated-measures analysis after adjustment for Intelligence Quotient as well as baseline value on any particular task.

Longitudinal comparisons

Ferritin responders in our paradigm were defined as women who showed a significant rise in serum ferritin from baseline to the end of the study that was greater than the known biological within-subject day-to-day variability in ferritin (24). Hemoglobin responders were defined in the same way by using the known variability in hemoglobin (24). Those women who experienced a significant improvement in serum ferritin (ferritin responders, n = 66) also had improvements in the attention and learning domains and in the memory domain that were 5 and 7 times, respectively, the improvements in the women who did not experience a significant increase in ferritin (ferritin nonresponders, n = 47) (Figure 5). No significant relation between the size of the ferritin change and the size of the cognitive change was evident, although such a relation may be found with a larger sample size or a longer intervention duration. In contrast to the improvement in performance, the time necessary to complete the tasks did not differ significantly between the women classified as ferritin responders or nonresponders.

The opposite results were found for the hemoglobin responders. The women who showed a significant change in hemoglobin concentration (hemoglobin responders, n = 33) over the 16-wk study completed the attention and memory tasks significantly faster (P < 0.001 for both) than did the women who did not experience a change in hemoglobin (hemoglobin nonresponders, n = 80) (Figure 6). As with the ferritin responders, no detectable relation between the size of the hemoglobin change and the size of the cognitive change was evident, although such a relation may be found with a larger sample size or a longer intervention duration. With respect to the learning tasks, both the hemoglobin responders and nonresponders showed significantly faster completion times at the end of the study than at the beginning (Figure 6). This decrease in the time necessary to complete the tasks for the hemoglobin responders did not carry over into an improvement in the performance factor on any of the cognitive tasks.

DISCUSSION

This study is the first in this age group to systematically examine the effect of iron status and intervention on cognitive functioning. We specifically examined the effects of ID and IDA on 3 cognitive domains—attention, memory, and learning. Whereas 8 different tasks were administered to each woman, the current report focuses on the examination of the data at the domain level rather than at the task level. This approach has been proposed as a means of examining the relation between a nutrient deficiency and cognition (25). Penland (25) suggested, because of the many compensatory mechanisms that are available, seeking a consistent change in a class of measures that assesses a meaningful underlying function may be more useful than seeking changes on specific measures.

Examination of the data at the domain (ie, attention, memory, and learning) level as well as across all domains found greater inefficiency in information processing in those women who were iron deficient than in those with normal iron status. The 3 domains that we tested were affected in a similar way. That is, with increasing severity of ID, cognitive performance decreased, whereas the time needed to complete the tasks increased. We also showed that administration of iron sulfate and an improvement in iron status result in an improvement in both performance of and the time necessary to complete learning, memory, and attention tasks. The current report documents the more “global” relations, but future reports will present data on the dimensions of cognition that are specifically altered by dietary ID. It is important to note that many factors influence cognition. One major factor known to be related to cognition is SES, with those from a socially disadvantaged background generally having lower cognitive performance than those from a more advantaged background. Most of the women in the current study were from a middle to upper-middle SES, and no differences were found between the groups with respect to SES. Other variables that were considered as possible confounders in the current study but that had no effect on our findings were age, grade point average, and timing of menstrual cycle in relation to when the cognitive tasks were completed. Whereas it is impossible to measure every potential confounder, the groups in the current study did not differ significantly with respect to any of the confounders measured. Therefore, we have a high level of confidence that our findings of changes in cognition are in fact due to the changes in iron status experienced by these women.

The decreased performance in memory found in the current study is consistent with the observations of Bruner et al (9) of
alterations in memory in iron-deficient adolescent girls. However, their study did not have the capacity to differentiate between changes in anemia and changes in body iron stores because that was not part of the study design. In the current study, ID has a separate effect from anemia because these are 2 discrete factors in the statistical analyses, which are separated statistically from each another. The factor termed storage was related to the performance component of the cognitive tasks as opposed to the time component. This statistical factor contained the variables of sTfR, body iron, TIR index, and ferritin that are normal biomarkers for the variation in iron status preceding the emergence of IDA if there is a negative iron balance. When we focused on just one of these variables, ferritin, it became clear that the women who improved their ferritin status showed improvements in performance on attention, memory, and learning tasks, but that the time to complete the task was unaffected. This relation of changes in ferritin concentrations and in cognitive performance is a highly important relation because it shows that persons do not have to be anemic to have alterations in attention, memory, and learning.

The relation of variation in anemia factor and hemoglobin was of course also explored in both the cross-sectional and the longitudinal analyses. The anemia factor was significantly related to speed of processing in the cross-sectional analysis, and, more specifically, a change in hemoglobin was significantly related to improvement in the speed of processing for the attention and memory tasks in the longitudinal analysis. By utilizing known day-to-day variation in biology for hemoglobin and ferritin to classify women as responders and nonresponders, we were able to examine the strength of association between anemia (hemoglobin) or ID (ferritin) and changes in both the speed of processing and the performance. When examining change, it was not possible to use the factors (as was done in the cross-sectional analyses), because biologic variation in the factors is unknown. Nonetheless, the consistency of the relation between hemoglobin and speed and that between ferritin and performance in both the cross-sectional and longitudinal data analyses give reassurance that our findings are not spurious. These findings are evidence that ID without anemia affects “how well” we do simple and complex cognitive tasks, whereas anemia affects “how fast” we complete those tasks. The functional benefit of iron therapy for the hemoglobin responders compared with that for the ferritin responders differs, which implies different but possibly overlapping causalities. The animal studies that examine changes in brain iron and neurotransmitter metabolism provide strong support for the concept that brain regional responses to iron therapy exist (4).

Whereas a few existing reports document the relation between iron status and cognition in premenopausal women (26–32), almost all of these studies are confounded by other variables (eg, pregnancy, weight loss, and dialysis). Nevertheless, most of the findings are in agreement and provide us with data with which we can compare our results. With respect to observational studies, Kretsch et al (27) reported a correlation of both hemoglobin and transferrin saturation with sustained attention in obese dieting women, whereas Foley et al (28) found a correlation between iron concentrations (ie, zinc protoporphyrin) and spatial performance in male and female undergraduate students. Our observation of a significant difference in the attention domain between iron-sufficient and iron-deficient women is in agreement with these observations. Positive cognitive effects of iron treatment have also been reported in adults. Groner et al (26) observed an improvement in short-term memory with iron supplementation of young pregnant women, whereas several other investigators reported an improvement in quality of life and cognitive functioning as a result of the normalization of iron status via erythropoietin therapy (29–32). One study (32) reported significant improvements in IQ, concentration, speed of information processing, and memory after partial correction of anemia in patients treated with recombinant human erythropoietin. Whereas the abovementioned studies may provide a comparison, the many confounders associated with the treatment of those subjects precludes us from concluding that the improvement in hemoglobin or hematocrit was the sole cause of the improvements seen in cognition. Our data are significant in that they show that normalization of iron status affects mental functioning in otherwise healthy adult women. Thus, the effects of ID on mental functioning are not limited to the early stages of development.

Whereas categorizing persons as iron sufficient or as having ID or IDA is traditionally done through the use of set cutoffs, we felt that this method limited our data; we therefore decided to treat our iron status variables as continuous. This was done by using the hematologic factors, each of which represented a more robust measure than any individual iron status variable and were also the result of a data reduction strategy, thereby eliminating problems with redundant variables and decreasing the probability of type 1 errors. Consequently, sorting the data by the storage factor allowed us to parse out the relation between a deficit in storage iron (and not necessarily other indicators of iron status) and cognition, as compared with sorting the data by the anemia factor, which allowed for the understanding of the relation between those with the most severe anemia and cognition. Specifically, we were able to show that low concentrations of the storage factor were related to performance, whereas low concentrations of the anemia factor were related to the time necessary to complete the specific tasks. We conclude that treating the data as continuous allowed for the detection of subtle cognitive changes that were overlooked when iron status was treated as categorical.

We have also shown that running the longitudinal analyses with women classified as responders or nonresponders is much more informative in efforts to detect cognitive changes than is simply running the analyses with women classified according to baseline groups. The observed association between changes in ferritin and changes in performance strongly suggests that brain ID is causally related to these changes in cognitive performance. The neurologic underpinnings of these findings are uncertain, but it is possible to make a few educated guesses. Animal models show that ID in postweaning life alters brain iron content, biochemical functioning, and neurotransmitter metabolism (4). The aspects that are not dependent on age or development are brain iron and biochemistry, brain energy metabolism, and perhaps resultant cell functioning. Whereas researchers have documented altered transmission in auditory and visual systems in infants with ID (33, 34), similar measurements are lacking in adults. Adult patients with RLS do, however, have a decreased brain iron content, and strong evidence exists that striatum-based dopamine biology is altered (35). Indeed, these patients have dramatic alterations in periodic limb movements that are often responsive to either L-dopamine treatment, iron therapy, or both. The current study subjects did not participate in any biologic measurements of
brain functioning, although previous studies with adults did show asymmetry of electroencephalograms with ID (36, 37).

One neurotransmitter system, dopamine, has been heavily examined in relation to brain ID, and it is well established that dopamine is implicated in memory, learning, and attention as well as in motor control, hormonal regulation, stress responsiveness, addiction, and emotional affect (38). Humans with attention deficit disorder or attention deficit hyperactivity disorder often show improvement in attentional performance when methylphenidate is given (39). This is a multifunctional drug, but its primary target is the dopaminergic system. Studies of pharmacologic challenges in humans found that dopaminergic agonists facilitated cognitive performance (40, 41) and dopaminergic antagonists impaired cognitive performance (42). Studies in the literature also examined the relation between cognitive function deteriorations seen with advancing age and the declines in the nigrostriatal dopamine system across the adult life span. These reports document a strong relation between D2 receptor binding in the striatum and cognitive performance that persists after control for age (43). This is consistent with a change in D2 receptor expression resulting from dietary ID in rodents (44). Other evidence of biochemical abnormalities in iron-deficient adults include increased concentrations of catecholamines in plasma and urine and decreased concentrations of thyroid hormones; both of these abnormalities are normalized after iron therapy (45–47). Whereas plasma and urine measurements are quite distant from brain activity, they do show that IDA can alter neurotransmitter metabolism in adults. Other literature regarding brain ID points to effects on gamma-aminobutyric acid and serotonin metabolism as well as on fundamental cellular bioenergetics (48). It would be speculative for us to suggest any particular causal biochemical pathway at this time; suffice it to point out that numerous feasible possibilities do exist. Whereas most of the evidence to date has been collected in animals, it is hoped that recent advances in technology will serve to elucidate the mechanisms by which iron status affects cognition in humans.

It is important to place these highly artificial cognitive tasks within the framework of functioning in everyday “real life” tasks. We show alterations in memory, learning, and attention with ID. Throughout the day, most persons will be required to attend to various situations, to remember, and even to learn certain information. In some situations, multitasking is expected and may be required. If a person has a deficit in attention, memory, or learning accuracy because he or she have depletion of essential body iron pools, that person’s ability to interact with the world at large will be lessened. This impairment may have negative consequences not only for the iron-deficient person but also for those around that person. An example is our recent demonstration that mother-child interactions are negatively affected when the mother is iron deficient (49, 50).

In conclusion, by using a conceptual model of adult intellectual abilities that encompasses a multitasking approach, we showed a relation between iron status and information processing in adult women of reproductive age. Future studies should be conducted to replicate these findings and to expand the findings to an understanding of how these cognitive deficits affect everyday life. This study furthers our understanding of the consequences of ID in adults and challenges the traditionally held viewpoint that ID does not have functional consequences until it has reached the level of anemia. Given these findings, better iron status surveillance practices are encouraged to identify persons who may be at risk of cognitive deficits.

We thank the women who agreed to participate in this study; the medical and nursing staff at the Pennsylvania State General Clinical Research Center who performed the blood draws and reviewed each subject’s medical history; Carrie Hagedus for her help with recruiting, analysis of the blood samples, and administration of the cognitive tasks.

Both authors were responsible for designing the experiment, analyzing the data, and writing the manuscript; LEM-K was responsible for collecting the data. Neither author had a personal or financial conflict of interest.

REFERENCES

19. Hollingshead AB. Two-factor index of social position. New Haven, CT: Department of Sociology, Yale University, 1957.


Maternal intake of vitamin D during pregnancy and risk of recurrent wheeze in children at 3 y of age\textsuperscript{1,2,3}


ABSTRACT

Background: Vitamin D deficiency and asthma are common at higher latitudes. Although vitamin D has important immunologic effects, its relation with asthma is unknown.

Objective: We hypothesized that a higher maternal intake of vitamin D during pregnancy is associated with a lower risk of recurrent wheeze in children at 3 y of age.

Design: The participants were 1194 mother-child pairs in Project Viva—a prospective prebirth cohort study in Massachusetts. We assessed the maternal intake of vitamin D during pregnancy from a validated food-frequency questionnaire. The primary outcome was recurrent wheeze, ie, a positive asthma predictive index (≥2 wheezing attacks among children with a personal diagnosis of eczema or a parental history of asthma).

Results: The mean (±SD) total vitamin D intake during pregnancy was 548 ± 167 IU/d. By age 3 y, 186 children (16%) had recurrent wheeze. Compared with mothers in the lowest quartile of daily intake (median: 356 IU), those in the highest quartile (724 IU) had a lower risk of having a child with recurrent wheeze [odds ratio (OR): 0.39; 95% CI: 0.25, 0.62; \(P\) for trend < 0.001]. A 100-IU increase in vitamin D intake was associated with lower risk (OR: 0.81; 95% CI: 0.74, 0.89), regardless of whether vitamin D was from the diet (OR: 0.81; 95% CI: 0.69, 0.96) or supplements (OR: 0.82; 95% CI: 0.73, 0.92). Adjustment for 12 potential confounders, including maternal intake of other dietary factors, did not change the results.

Conclusion: In the northeastern United States, a higher maternal intake of vitamin D during pregnancy may decrease the risk of recurrent wheeze in early childhood. \textit{Am J Clin Nutr} 2007;85:788–95.

KEY WORDS Vitamin D, pregnancy, dietary intake, childhood wheeze, asthma

INTRODUCTION

Vitamin \(D_3\) (cholecalciferol) is a vital nutrient available from food sources (eg, fortified milk) and nutritional supplements (1). The skin can also make vitamin D when exposed to sunlight. Recent studies suggest that vitamin D may have value in preventing or treating cancer and autoimmune diseases (2, 3). It has also become clear, especially in the northeastern United States (4, 5), that a large proportion of Americans have an inadequate vitamin D intake, as reflected by serum 25-hydroxyvitamin D concentrations (6).

Another public health problem in the northeastern United States is asthma. For example, the prevalence rates of asthma in New England are the highest in the nation (7). The national prevalence of asthma has increased from ≈3% in the 1970s to ≈8% in recent years (8). The onset of asthma occurs before 6 y of age in 80–90% of cases; 70% of cases occur before 3 y of age (9, 10). Asthma has multifactorial origins, but major risk factors have proven elusive. Some investigators have focused on dietary origins, but studies to date are mostly cross-sectional and have yielded inconsistent results (11). Although vitamin D is known to have important effects on immunologic function (12), the role of vitamin D on risk of asthma is unknown. The role of maternal diet on risk of asthma in offspring is a particularly intriguing topic given the growing evidence on the developmental origins of health and disease (13, 14) and the early age of asthma onset (15, 16).

We hypothesized that higher vitamin D intakes are protective against asthma in populations with inadequate vitamin D intakes. We formulated this hypothesis after noting the similarity between risk factors for vitamin D insufficiency and for asthma. In addition to the regional similarities noted above, vitamin D insufficiency is more common among African Americans (17) and among the obese (18). Temporal trends in the adoption of sunscreen use (19) and in reductions in milk intake (20) also support the hypothesized link between vitamin D and asthma. The purpose of the present study was to determine, among mothers living in the northeastern United States, whether a higher maternal intake of vitamin D intake during pregnancy is associated with a lower risk of asthma in children at 3 y of age.

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SUBJECTS AND METHODS

Study design and subjects

Project Viva is a prospective cohort study examining prenatal factors in relation to outcomes of pregnancy and child health. Participants were recruited at 8 obstetric offices of Harvard Vanguard Medical Associates, a large multispecialty urban-suburban group practice in eastern Massachusetts. At the first study visit, directly after the woman’s initial clinical prenatal visit, we obtained informed consent, administered a brief interview, and provided a take-home self-administered questionnaire. At the second study visit, at 26 to 28 wk of gestation, we again administered a brief interview and provided a questionnaire. Project Viva participants gave birth at Brigham and Women’s Hospital or Beth Israel Deaconess Medical Center in Boston. Within 3 d after delivery, we interviewed the mothers. We reassessed mothers and children at 6 mo, 1 y, and annually thereafter. At the time of this analysis, the visits at age 3 y were almost complete.

Exclusion criteria included multiple gestation (eg. twins), the inability to answer questions in English, plans to move out of the area before delivery, and a gestational age ≥22 completed weeks at the initial prenatal clinical appointment. Additional details of recruitment and follow-up were presented elsewhere (21). Of the 2128 infants delivered in Project Viva, we excluded 228 because of missing diet assessment data from the first and second trimesters and an additional 37 with a gestational age of <34 completed weeks. Mothers of 368 of the remaining 1863 participants did not provide informed consent for child follow-up through age 3 y. Of the remaining 1495 infants, 76 were lost to follow-up and 225 have not yet exited the 3-y time window. The final sample for analysis consisted of 1194 participants. This sample appears representative of the overall study population. The human subjects committees of Harvard Pilgrim Health Care, Brigham and Women’s Hospital, and Beth Israel Deaconess Medical Center approved the study protocols.

Measurements

We obtained data directly from the participants and from medical records, as detailed previously (21). Briefly, maternal diet assessments at both the first and second visits were based on a validated 166-item semiquantitative food-frequency questionnaire (FFQ) (22). We slightly modified the FFQ for use in pregnancy from the FFQ used in the Nurses’ Health Study and other cohort studies (23). Modifications for use in pregnancy included changing the time referent, beverage section, and vitamin and supplement assessment. The FFQ used at the first visit reflected intakes in the first trimester; the time referent was “during this pregnancy.” In addition, we collected information on beverage intake during 2 specified time periods: before and after the participant learned that she was pregnant. To assess vitamin and supplement intakes during the first trimester, we administered a separate interview that assessed dose, duration, and brand or type of multivitamin, prescribed prenatal vitamin, and supplements. The FFQ used at the second visit (26–28 wk of gestation) reflected intakes during the second trimester; the time referent was “during the past 3 mo.” The second trimester instrument was the same as that used in the first trimester except that we assessed the use of vitamins and supplements as part of the self-completed FFQ and we collected beverage information during the one time period.

To calculate nutrient intakes, we used the Harvard nutrient composition database, which contains food-composition values from the US Department of Agriculture, and other sources (24). We calculated vitamin D intake for each FFQ, and the mean of these 2 values was the assigned exposure for each woman during pregnancy (25). We used a similar approach for retinol and calcium intakes. For the analyses based on foods rather than nutrients, we focused on milk (servings/d)—the major contributor to vitamin D intake in the diet. We also examined foods with possible effects on asthma risk (11, 26): fruit and vegetables (servings/d) and fish (servings/wk).

At the first visit in early pregnancy, in addition to diet assessment, we obtained information on maternal age, race-ethnicity, household income, education, marital status, last menstrual period (LMP), height, prepregnancy weight, gravidity, and history of atopic disorders. At the second visit in midpregnancy, we updated many of these variables. We calculated maternal prepregnancy body mass index as weight (in kg) divided by the square of height (in m). We calculated gestational age using the mother’s reported LMP, or from second trimester ultrasound if they differed by >10 d. From the hospital vital statistics record, we abstracted infant birth weight. We determined a birth weight–for–gestational age z value (fetal growth) by using US reference data (27). At the 6-mo and 1-y visits, we asked about infant feeding, vitamin supplements, and household exposures. At the 2-y visit, we also used a validated FFQ to assess the children’s vitamin D intakes (28).

Our primary outcome was based on the Asthma Predictive Index (29). We assessed wheeze status initially by asking 2 questions at each of the 3 annual visits: 1) Since your child was born (was 12 mo old, was 2 y old) has he or she ever had wheezing (or whistling in the chest)? , and 2) Since your child was born (was 12 mo old, was 2 y old), how many attacks or episodes of wheezing has he or she had? We defined recurrent wheeze as ≥2 wheezing attacks (summed from the 1-, 2-, and 3-y annual questionnaires) in children with a personal diagnosis of eczema or parental history of asthma.

Secondary wheeze outcomes included the larger group of children with “any wheeze” (ie, any report of wheezing during the first 3 y of life). Also, at the other end of the wheezing spectrum, we looked at 2 subsets of children with recurrent wheeze: 1) those who also had doctor-diagnosis of asthma (by maternal report), and 2) those with ≥4 wheezing attacks (summed over the annual questionnaires). These 2 subgroup analyses excluded subjects with an intermediate phenotype so that children who satisfied the more stringent case definitions were compared with children without any wheeze.

Finally, we examined 2 related outcomes: respiratory infection and eczema. We defined respiratory infection as a positive response to ≥1 of 3 questions on any of the annual questionnaires: Since your child was born (was 12 mo old, was 2 y old), have you been told by a health professional that he or she has bronchiolitis, pneumonia, bronchitis, croup, or any other respiratory infection? We defined eczema as a positive response to one question on any of the annual questionnaires: Have you ever been told by a health care professional that your child has eczema?

Data analysis

To assess the associations between vitamin D intake and clinical outcomes, we used multiple logistic regression models. We
RESULTS

The mean (±SD) age of mothers at enrollment was 32.5 ± 4.9 y. Most of the mothers (72%) had at least a college education, and 65% lived in households with an annual income of >$70 000. The mean (±SD) prepregnancy BMI was 24.5 ± 5.1, and 10% of the mothers smoked during the index pregnancy. Approximately half of the children were male (51%) and 74% were white. Their mean (±SD) birth weight was 3.51 ± 0.52 kg, and the mean (±SD) gestational age was 39.6 ± 1.5 wk. Children were breastfed for a mean (±SD) of 6.4 ± 4.5 mo, and 53% were born into homes with other children aged <12 y. Of the mothers, 31% had either asthma (17%) or eczema (20%); 26% of the fathers had either asthma (14%) or eczema (15%).

The mean (±SD) total vitamin D intake during pregnancy was 548 ± 167 IU/d, with an average of 225 IU from food and 319 IU from supplements. Maternal intake of vitamin D during pregnancy was <400 IU/d in 19% of women. Milk was the primary food contributor to vitamin D intake during pregnancy, accounting for 53% of intake; other substantial contributors were fish (18%) and cold cereal (9%).

Maternal vitamin D intake was associated with several factors that might affect risk of asthma (Table 1). Mothers with a higher intake of vitamin D were slightly older, less overweight, of higher socioeconomic status, less likely to smoke during pregnancy, and more likely to have a personal history of eczema. They consumed a little more fish but their intake of fruit and vegetables did not differ. Maternal vitamin D intake was not associated with conception during the winter months (ie, fall birthday), sex, birth weight, or gestational age. However, children of women with a higher maternal intake of vitamin D were more likely to be born to white mothers, to have been breastfed longer, to take a vitamin supplement in the first 6 mo of life, and to consume more vitamin D from foods at age 2 y. For the children, the mean (±SD) vitamin D intake from foods was 249 ± 101 IU/d; milk contributed 71% of vitamin D, fish 1%, and cold cereal 17%. The home environment of mothers with a high vitamin D intake was characterized by less passive smoke exposure and fewer siblings aged <12 y, but no difference in exposure to pets or other common allergens was found.

The unadjusted inverse linear association between maternal vitamin D intake and risk of recurrent wheeze by exposure to pets or other common allergens was found in Figure 1. Because individual observations would otherwise be difficult to distinguish, we added random noise to the display of each observation. The smoothed line shows the approximate probability of recurrent wheeze for each observed value of vitamin D intake. This strong inverse association is confirmed in Table 2 (P for trend <0.001). Compared with mothers in the lowest quartile of daily intake (median within quartile: 356 IU), those in the highest quartile (median: 724 IU) had a lower risk of having a child with recurrent wheeze at age 3 y (OR: 0.39; 95% CI: 0.25, 0.62). The strong inverse association between maternal vitamin D intake and recurrent wheeze was robust to controlling for a variety of potential confounders, including traditional asthma risk factors and putative dietary risk factors such as fish, fruit, and vegetables (Table 2). Further adjustment for a variety of micronutrients (eg, vitamin C, vitamin E, and zinc) did not affect the results (data not shown). In contrast with the decreased risk of recurrent wheeze, maternal intake of vitamin D was not associated with risk of respiratory infections or early childhood eczema (Table 2).

To further explore these results, we examined risk according to vitamin D intake, both during pregnancy and over the first 2 y of life. As shown in Table 1, women with a higher vitamin D intake during pregnancy were more likely to have children who used vitamin supplements and ate more foods with vitamin D. Nevertheless, the association of maternal vitamin D intake with recurrent wheeze (Table 2; model 2) did not change with further control for the child’s use of a vitamin supplement at age 6 mo (OR: 0.39; 95% CI: 0.23, 0.67; P for trend <0.001) or for the child’s vitamin D intake from foods at 2 y (OR: 0.34; 95% CI: 0.20, 0.60; P for trend <0.001). This issue is addressed in another way in Figure 2: the mothers and children are stratified by the vitamin D intake of each group, with high-low cutoffs set at 400 IU for mothers and 200 IU for children. The data in the figure show that the decreased risk of recurrent wheeze was largely, if not entirely, due to a high maternal intake of vitamin D during pregnancy.

The risk of recurrent wheeze with maternal vitamin D intake, expressed as a 100-IU/d increase rather than in categories, is shown in Table 3. The results closely resemble the strong inverse association shown in Table 2. Moreover, the results were similar for vitamin D from food only and for vitamin D from supplements only. Further adjustment for race-ethnicity and college education did not change the inverse association between maternal vitamin D intake and risk of recurrent wheeze (OR: 0.81; 95% CI: 0.72, 0.90). We also examined the potential effect of 2 dietary factors correlated with vitamin D intake: retinol (r = 0.53) and calcium (r = 0.57). Neither nutrient confounded the strong inverse association between maternal vitamin D intake and risk of recurrent wheeze (Table 3).

To further explore the spectrum of wheezing illnesses of childhood, we looked at the relation of maternal vitamin D intake with risk of any wheeze (n = 416 cases) and with subsets of the primary outcome: recurrent wheeze with doctor-diagnosed asthma (n = 132 cases) and recurrent wheeze with ≥4 wheezing episodes (n = 86 cases). For each 100-IU increase in maternal vitamin D intake, we observed a somewhat weaker but still significantly lower risk of any wheeze (OR: 0.89; 95% CI: 0.82, 0.97). The inverse association appeared stronger for the most stringent wheeze outcomes: children with recurrent wheeze and doctor-diagnosed asthma (OR: 0.82; 95% CI: 0.71, 0.93) or recurrent wheeze with ≥4 wheezing episodes (OR: 0.79; 95% CI: 0.67, 0.93). All of these values were adjusted for the 12 factors in model A (Table 3).
As noted earlier, a major dietary source of vitamin D is fortified milk. Accordingly, we examined the association between milk intake by mothers during pregnancy and risk of clinical outcomes in their children. Compared with mothers who did not drink milk (who had a median vitamin D intake of 479 IU from other foods and supplements), and after adjustment for 12 factors, risk of recurrent wheeze was lowest among those drinking 1–1.9 cups/d (OR: 0.35; 95% CI: 0.16, 0.80). The associations were less extreme for those drinking <1 cup/d (OR: 0.57; 95% CI: 0.27, 1.24) or ≥2 cups/d (OR: 0.45; 95% CI: 0.20, 1.02). The overall P value for milk in this multivariate model was 0.046, whereas the P for linear trend (across categories of increasing milk intake) was 0.13.

Finally, we explored effect modification of the association of maternal intake of vitamin D with risk of recurrent wheeze. Only season of LMP showed a likely interaction (P = 0.06). Maternal intake of vitamin D was associated with a lower risk of recurrent wheeze when the LMP was in the winter. The multivariate OR per 100-IU/d increase was 0.62 (95% CI: 0.47, 0.83) for LMP in winter compared with 0.85 (95% CI: 0.75, 0.97) in other seasons.

DISCUSSION

In this prospective cohort study of almost 1200 mother-child pairs from the northeastern United States, a higher maternal intake of vitamin D during pregnancy was associated with a
lower risk of recurrent wheeze in children 3 y of age. The inverse association was independent of many potential confounders, including adjustment for fish intake, fruit and vegetable intake, early use of a vitamin supplement, and child’s intake of vitamin D at age 2 y. The association was present for vitamin D from either foods or nutritional supplements. In addition, among children conceived in the winter months and therefore born in the fall (a time of low ultraviolet light B exposure), the inverse association between maternal intake of vitamin D and risk of recurrent wheeze was stronger.

In recent years, vitamin D has been a focus of growing interest in public health nutrition (2, 3). Vitamin D insufficiency is common in the United States, especially in northern latitudes (4, 5). This insufficiency is multifactorial but probably stems from a combination of decreased dietary intake of vitamin D (eg, from fortified milk or fish) and decreased sun exposure (eg, because of lifestyle choices or increased use of sunscreen). Indeed, northern populations receive too few ultraviolet light B rays between November and March to generate sufficient vitamin D in the skin (4), and studies suggest that one cannot drink enough milk to overcome this shortfall (30). The reemergence of childhood rickets in recent years (31) has led the American Academy of Pediatrics to recommend vitamin D supplementation for high-risk

![FIGURE 1. Unadjusted inverse linear association between maternal vitamin D intake and risk of recurrent wheeze in offspring. The top band of dots (Yes) represents children who developed recurrent wheeze, and the bottom band of dots (No) represents children who did not develop recurrent wheeze. The vertical lines represent percentiles of maternal vitamin D intake.](image)

![FIGURE 2. Risk of recurrent wheeze stratified by vitamin D intake. (Groups are split at 400 IU vitamin D for mothers during pregnancy and at 200 IU vitamin D for children at age 2 y.) Odds ratios are adjusted for the 12 factors in multivariate model 2 (see Table 2). In a formal test for interaction, the main effect of maternal vitamin D intake was significant \((P < 0.001)\), but the main effect of child vitamin D intake and the interaction term for maternal-child vitamin D intake were not \((P > 0.80 \text{ for both})\).](image)

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### TABLE 2
Odds ratios (and 95% CIs) for maternal intake of vitamin D during pregnancy and risk of clinical outcomes in children at 3 y of age \((n = 1194\) mother-infant pairs)

<table>
<thead>
<tr>
<th>Quartile of vitamin D intake by mother during pregnancy</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>(P) for trend(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D intake (IU)</td>
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<td></td>
<td></td>
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<tr>
<td>Median</td>
<td>356</td>
<td>513</td>
<td>603</td>
<td>724</td>
<td></td>
</tr>
<tr>
<td>No. of subjects</td>
<td>298</td>
<td>299</td>
<td>299</td>
<td>298</td>
<td></td>
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<tr>
<td>Recurrent wheeze ((n = 186) cases)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Unadjusted</td>
<td>1.00</td>
<td>0.55 (0.36, 0.84)</td>
<td>0.55 (0.36, 0.83)</td>
<td>0.39 (0.25, 0.62)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Multivariate model 1(^2)</td>
<td>1.00</td>
<td>0.49 (0.30, 0.80)</td>
<td>0.56 (0.34, 0.92)</td>
<td>0.41 (0.24, 0.70)</td>
<td>0.001</td>
</tr>
<tr>
<td>Multivariate model 2(^3)</td>
<td>1.00</td>
<td>0.47 (0.28, 0.77)</td>
<td>0.54 (0.33, 0.89)</td>
<td>0.38 (0.22, 0.65)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Respiratory infection ((n = 540) cases)</td>
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<tr>
<td>Unadjusted</td>
<td>1.00</td>
<td>0.68 (0.48, 0.96)</td>
<td>0.92 (0.65, 1.30)</td>
<td>0.68 (0.48, 0.96)</td>
<td>0.09</td>
</tr>
<tr>
<td>Multivariate model 1(^2)</td>
<td>1.00</td>
<td>0.73 (0.51, 1.06)</td>
<td>1.01 (0.70, 1.45)</td>
<td>0.74 (0.51, 1.08)</td>
<td>0.28</td>
</tr>
<tr>
<td>Multivariate model 2(^3)</td>
<td>1.00</td>
<td>0.74 (0.51, 1.06)</td>
<td>1.01 (0.70, 1.46)</td>
<td>0.75 (0.52, 1.09)</td>
<td>0.32</td>
</tr>
<tr>
<td>Eczema ((n = 428) cases)</td>
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<td></td>
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<tr>
<td>Unadjusted</td>
<td>1.00</td>
<td>1.00 (0.71, 1.39)</td>
<td>1.03 (0.74, 1.44)</td>
<td>1.10 (0.79, 1.53)</td>
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<td>1.00</td>
<td>0.88 (0.60, 1.27)</td>
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</tr>
<tr>
<td>Multivariate model 2(^3)</td>
<td>1.00</td>
<td>0.86 (0.59, 1.25)</td>
<td>0.93 (0.64, 1.36)</td>
<td>0.92 (0.63, 1.35)</td>
<td>0.75</td>
</tr>
</tbody>
</table>

\(^1\) From logistic regression models using the vitamin D variable; the median value within each quartile was used as the predictor variable.

\(^2\) Adjusted for sex, birth weight, income, maternal age, maternal prepregnancy BMI, passive smoking exposure, breastfeeding duration at 1 y, number of children <12 y of age in household, maternal history of asthma, and paternal history of asthma. For eczema analyses, the model adjusted for parental history of eczema instead of asthma.

\(^3\) Adjusted for the 10 factors in model 1 plus intakes of fish and fruit and vegetables.
groups (32). Most nutritionists rely on the 1997 Institute of Medicine recommendations (33), but a growing number are recommending that high-risk populations take a daily supplement of 800 to 1000 IU (34).

At present, very little is known about how vitamin D affects the lungs. Some studies report findings that are in general agreement with ours. For example, Black and Scragg (35) recently reported cross-sectional data from the third National Health and Nutrition Examination Survey and found that serum 25-hydroxyvitamin D was inversely associated with forced expiratory volume in 1 second in the US general population. The authors and the accompanying editorial (36) called for studies to determine whether vitamin D is of any benefit in patients with respiratory disease. Concurrent with this population-based study, Xystrakis et al (37) in London administered vitamin D to a small group of healthy persons and steroid-resistant asthmatic patients and found that the intervention enhanced subsequent responsiveness to dexamethasone for induction of interleukin (IL)-10. The authors suggested that vitamin D could potentially increase the therapeutic response to glucocorticoids in steroid-resistant asthma patients.

In contrast, a birth cohort in northern Finland reported that regular vitamin D supplementation in the first year of life increased the risks of developing atopy, allergic rhinitis, and asthma by age 31 y (38). The authors, however, collected no information on maternal intakes of the vitamin, and control for major confounders was limited. Furthermore, recall bias may have affected the ascertainment of early life asthma and allergies. Although these results are consistent with historical concerns about the potential adverse effects of milk intake on asthma and allergic diseases, most studies do not support this hypothesis (39). Indeed, the few epidemiologic studies that have examined the issue have reported an inverse association between milk intake and risk of asthma in young children (40, 41).

Laboratory research suggests several potential mechanisms for how vitamin D can affect risk of asthma. For example, several recent genetic association studies (42–45) suggest that polymorphisms of the VDR gene are related to obstructive pulmonary disease. Other proposed mechanisms include modulation of antigen-presenting cells, such as macrophages (46, 47), and the generation of regulatory T cells (48, 49) that express potentially inhibitory cytokines (IL-10 and transforming growth factor β), and the ability to potentiate inhibit antigen-specific T cell activation (50). A murine model of pulmonary eosinophilic inflammation showed that vitamin D supplementation of adult mice led to changes in cytokines, immunoglobulin E concentrations, and airway eosinophilia during allergen sensitization (51). Although many laboratory studies suggest that vitamin D induces a shift in the balance between T helper subset 1 (Th1)– and Th2-type cytokines toward Th2 dominance (12), Pichler et al (52) found that in CD4+ and CD8+ human cord blood cells, vitamin D inhibits not only IL-12–generated interferon γ production but also suppresses IL-4 and IL-4–induced expression of IL-13. If a shift away from the fetal Th2-type pattern in humans is linked with asthma protection, our maternal intake findings are consistent with the cytokine findings of Pichler et al. Thus, the differences between the studies on the Th1-Th2 dominance may lie in the timing of exposure of the cells to vitamin D (ie, prenatal versus postnatal); the response of naïve T cells to vitamin D exposure may differ from that of mature cells when exposed to vitamin D (53). Another possibility is that the association depends on the vitamin D status of the individual. In other words, lower vitamin D intakes (eg, to replete a deficiency state) may have different consequences than relatively high-dose supplementation, whereas an excess of vitamin D may indeed have opposite effects. These hypotheses merit further investigation.

Our study has several potential limitations. The early age of outcome measurement limits our ability to generalize these findings from recurrent wheeze to actual asthma. Many children who wheeze before 3 y of age have transient episodes and do not go on to have asthma (54). However, the chosen 3-y outcome carries an almost 10-fold increased risk of developing asthma by age 6 y (29). Continued follow-up of our birth cohort will determine whether the findings translate to a decreased risk of asthma later in childhood.

Another limitation is that all participants came from Massachusetts, were predominantly white, and were of higher socioeconomic status; the generalizability of the observed associations merits further study. However, on the basis of a growing body of literature on vitamin D, we anticipate stronger associations among African Americans and Hispanics living in the northern United States. Large interindividual differences in time...
spent outdoors and in sunshine use will complicate the generalizability of these diet-asthma associations to regions with more sunlight exposure.

In summary, we found a strong inverse association between maternal intake of vitamin D during pregnancy and risk of recurrent wheeze in children at 3 y of age. The protective association was present for vitamin D from both diet and nutritional supplements and was observed in the context of apparently adequate intakes of vitamin D (33) for most women in this northeastern US population. Pregnant and lactating women are known to be at higher risk of vitamin D deficiency (55), and our findings provide additional support for efforts to improve the nutritional status of this population, including recommended intakes of >400 IU/d. The primary implications of our findings are in the research arena, eg, the need for additional prospective studies to confirm or refute our vitamin D-asthma hypothesis. If others replicate our findings, we would support the initiation of randomized trials of vitamin D supplementation and was observed in the context of apparently adequate intakes of vitamin D for most women in this northeastern US population. Pregnant and lactating women are known to be at higher risk of vitamin D deficiency, and our findings provide additional support for efforts to improve the nutritional status of these populations, including recommended intakes of >400 IU/d. The primary implications of our findings are in the research arena, eg, the need for additional prospective studies to confirm or refute our vitamin D-asthma hypothesis. If others replicate our findings, we would support the initiation of randomized trials of vitamin D supplementation.

REFERENCES

41. Hijazi N, Abalkhail B, Seaton A. Diet and childhood asthma in a society
Maternal smoking is associated with decreased 5-methyltetrahydrofolate in cord plasma1–3

Ken D Stark, Robert J Pawlosky, Robert J Sokol, John H Hannigan, and Norman Salem Jr

ABSTRACT

Background: Maternal-fetal folate transport via the placenta has been shown to be concentrative. Exposure to cigarette smoke is associated with decreased maternal folate status through altered dietary intakes and possibly through nondietary mechanisms such as increased folate turnover. The effect of maternal smoking on fetal folate status has not been documented.

Objective: The objective was to determine the effect of maternal smoking on plasma 5-methyltetrahydrofolic acid (5-MTHFA) concentrations in umbilical cord blood.

Design: African American women were recruited from an antenatal clinic in Detroit, MI. Plasma 5-MTHFA concentrations were measured in maternal-umbilical cord pairings (n = 58). The participants completed a structured interview to determine demographic characteristics, including smoking.

Results: Concentrations of 5-MTHFA were significantly higher in venous cord plasma (16.8 ± 7.5 ng/mL) than in maternal plasma (13.0 ± 7.5 ng/mL) but remained associated (r = 0.60, P < 0.001) with each other. Cigarettes smoked by the mothers was negatively associated (r = −0.31, P = 0.019) with venous cord 5-MTHFA concentrations and remained so after control for maternal plasma 5-MTHFA and other variables. Venous cord plasma 5-MTHFA was significantly lower in smoking (15.1 ± 7.6 ng/mL; n = 32) than in nonsmoking (19.0 ± 7.0 ng/mL; n = 26) mothers.

Conclusions: Cord plasma 5-MTHFA concentrations were elevated relative to maternal blood, as expected, because the placenta is capable of concentrative folate transport to the fetus. The negative effect of maternal smoking on infant, but not on maternal, 5-MTHFA status indicates that maternal smoking may impair folate transport to the fetus. Am J Clin Nutr 2007;85:796–802.

KEY WORDS 5-Methyltetrahydrofolic acid, folate, folic acid, African American women, pregnancy, smoking, electrospray mass spectrometry, infants, umbilical cord, placenta

INTRODUCTION

Pregnant, inner-city African American women are at risk of consuming less than the recommended dietary allowance (RDA) of dietary folate and engage in lifestyle habits, such as smoking, that can further reduce maternal folate status (1). In the United States, population-wide serum and erythrocyte folate concentrations have increased with folic acid fortification, but concentrations remain lower in non-Hispanic black women than in other ethnic groups (2). Also, the incidence of infants born with neural tube defects has decreased less in non-Hispanic black populations than in Hispanic and non-Hispanic white populations (3). In Wayne County, MI, or in the state of Michigan, the incidence of congenital abnormalities of the central nervous system has not decreased after folate fortification (4). The adequacy of current levels of folic acid fortification is debatable, but clearly other factors must be considered (5).

The effect of cigarette smoking on pregnancy outcomes is complex because it is associated with other behaviors, including reduced micronutrient and increased alcohol intakes (6). Maternal smoking during pregnancy has been identified as the most important determinant of birth weight in developed countries (7) and may increase the risk of neural tube defects (8, 9), orofacial clefts (10, 11), and congenital heart defects (12). Adequate folate status is associated with a reduced risk of each of these outcomes (13–16). Folate intake, which is reduced with smoking (17), is the major determinant of blood folate concentrations; however, the negative association between smoke exposure and blood folate status persists after adjustment for folate intakes in studies before and after folic acid fortification (1, 18).

Smoking is associated with reduced circulating maternal folate concentrations (1, 6, 19). To our knowledge, there have only been 2 reports of the effects of maternal smoking on umbilical cord or infant folate concentrations, with both showing no effect (14, 20). No data exist on the effects of maternal smoking on umbilical cord folate concentrations in African American women. Maternal-to-fetal folate transfer is concentrative, as shown in perfused human placenta studies (21–23) and in maternal-umbilical cord comparisons (24, 25). The mechanism of placental folate transport is yet to be fully elucidated, but it

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appears that there is a high affinity folate receptor at the syncytiotrophoblast that mediates endocytosis at the microvillus membrane followed by efflux into fetal blood at the basolateral side by a reduced folate carrier (21). Maternal smoking can also alter placental development, induce placental hypoxia, and impair placental nutrient transport (6, 26).

The purpose of the present study was to examine the effects of maternal smoking at the time of the first prenatal visit on 5-methyltetrahydrofolic acid (5-MTHFA) concentrations in maternal plasma and umbilical cord plasma in an inner-city African American population at-risk for behaviors detrimental to health. Accurate and specific measurements of 5-MTHFA were made in maternal plasma, cord venous plasma, and cord arterial plasma by stable-isotope HPLC-mass spectroscopy-electrospray ionization (HPLC-MS-ESI) (27) and combined with detailed demographic, clinical, and behavioral data, including smoking, alcohol, and dietary intakes.

SUBJECTS AND METHODS

Subjects and sample collection

Pregnant African American women attending the Antenatal Clinic at Wayne State University (Detroit, MI) between February 1999 and January 2001 were recruited into the present study as described previously (1). All procedures and protocols received prior approval by the Wayne State University Human Investigations Committee, and informed consent was obtained during the initial clinical visit. Women with high-risk pregnancies, with known fatty acid metabolism disorders, with diabetes, and who developed gestational diabetes were excluded from the study. All of the infants in the present study were singleton and free of malformations as determined by dysmorphology and neurobehavioral testing procedures common in fetal alcohol syndrome screening (28).

A structured interview at the first antenatal visit determined eligibility and assessed demographic characteristics, alcohol intake, and smoking exposure (29, 30). In this interview, quantitative smoking (cigarettes smoked/d) was determined at the time of the interview and before pregnancy by maternal recall. Socioeconomic status was measured by using a modified Hollingshead index (31). Demographic and clinical characteristics were collected for 282 mother-singleton infant pairs at delivery. Nutrient intakes were assessed at the time of delivery as previously published (1, 30, 32) with a food-frequency questionnaire validated for nutrient and folate intakes in low-income pregnant women (33, 34). The US Department of Agriculture National Nutrient Database for Standard Reference, release 14, was used for quantification (35). Individual nutrient intakes were adjusted for total energy intake with the nutrient residual method (36) to reduce measurement error (37). Dietary folate equivalents (DFEs) were calculated as recommended by the Food and Nutrition Board in the Dietary Reference Intakes for folate (38): for natural food sources of folate, 1 μg folate = 1 μg DFE; for synthetic vitamin preparations, 1 μg folic acid = 2 μg DFE; and for mixed food and supplement products, μg DFE is calculated as food folate (in μg) × 1.7 + folic acid (in μg) × 1.7. All participants were advised about nutrient supplementation during pregnancy and received a prescription to obtain prenatal vitamins, but compliance with daily multivitamin use in urban African Americans is very low (39).

Maternal blood (15 mL) was collected shortly before infant delivery by venipuncture of the antecubital vein. At delivery, infant demographics were recorded and as much separate arterial and venous umbilical cord blood was collected as possible. Both the maternal and umbilical cord blood specimens were collected into heparinized tubes and kept cold (4 °C) until centrifugation (5 min, 2000 × g) to separate plasma and erythrocytes, and the plasma was frozen at −75 °C until analyzed.

Laboratory analyses

Plasma 5-MTHFA concentrations in maternal and umbilical venous and arterial samples were measured by HPLC-MS-ESI with an intrarassay CV of 5.3% and an interassay CV of 7.6% as described previously (27). Before extraction, 10 ng of 5-MTHFA (10 ng) was added to 0.5 mL plasma as an internal standard. The analyte was isolated by using solid-phase extraction (Strata phenyl column 100 mg/mL; Phenomenex, Torrance, CA), washed with 0.03 mol K2HPO4/L, and eluted with 0.5 mL of the HPLC mobile phase (acetonitrile:methanol:water, 26:14:60). Forty microliters of extract was injected onto a C18 HPLC column (150 × 4.6 mm; Phenomenex) with the use of a binary pumped Agilent 1100 HPLC (Palo Alto, CA) interfaced to an ion-trap MS (Finnigan LCQ San Jose, CA), and samples were analyzed by ESI in the positive ion mode.

Statistical analyses

Data were normally distributed as determined by the Kolmogorov-Smirnov procedure. Comparisons between maternal plasma 5-MTHFA, venous cord plasma 5-MTHFA, and arterial cord plasma 5-MTHFA were made by repeated-measures ANOVA; individual mean comparisons were made by using Tukey’s honestly significantly different (HSD) test. Maternal plasma 5-MTHFA and venous plasma 5-MTHFA were compared by paired t test. Bivariate associations of cigarettes smoked by the mothers before pregnancy and at the first prenatal visit, cigarettes smoked by the fathers, maternal plasma 5-MTHFA, maternal DFE intake, and venous cord plasma 5-MTHFA were determined by Pearson’s correlations (two-tailed). Plasma 5-MTHFA in smokers and nonsmokers at delivery were compared by independent t test for maternal and venous cord samples and by general linear models with various covariates. Associations with venous cord plasma 5-MTHFA were also evaluated by using multiple linear regression analyses. A parsimonious model with independent variables having P values <0.10 were included and a controlled model were generated. Variables were included based on information from the literature, influence on the model r value, and degree of collinearity with other variables. The consistency of the statistical results were examined after the exclusion of 2 participants who consumed a prenatal multivitamin that included folic acid regularly throughout pregnancy, as determined by dietary records and maternal 5-MTHFA. All statistical analyses were completed with SPSS for WINDOWS statistical software (release 11.5.1; SPSS Inc, Chicago, IL) with statistical significance inferred when P values were <0.05.

RESULTS

Maternal characteristics

Sample size in the present study was limited primarily by the difficulties in collecting adequate amounts of separate and unhemolyzed venous and arterial cord blood combined with sample
Characteristics and dietary intakes of pregnant African American women

<table>
<thead>
<tr>
<th></th>
<th>All subjects (n = 282)</th>
<th>Smokers (n = 32)</th>
<th>Nonsmokers (n = 26)</th>
<th>All subjects (n = 58)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (y)</td>
<td>24.8 ± 5.4</td>
<td>26.1 ± 5.9</td>
<td>23.8 ± 5.1</td>
<td>25.1 ± 5.6</td>
</tr>
<tr>
<td>Gestational age at 1st prenatal visit (wk)</td>
<td>16.3 ± 6.4</td>
<td>16.9 ± 6.9</td>
<td>17.2 ± 6.9</td>
<td>17.0 ± 6.8</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.66 ± 0.07</td>
<td>1.66 ± 0.06</td>
<td>1.66 ± 0.09</td>
<td>1.66 ± 0.07</td>
</tr>
<tr>
<td>Prepregnancy weight (kg)</td>
<td>74.3 ± 20.8</td>
<td>78.4 ± 21.7</td>
<td>75.8 ± 18.1</td>
<td>77.3 ± 20.1</td>
</tr>
<tr>
<td>Education (highest grade)</td>
<td>11.8 ± 1.4</td>
<td>11.7 ± 1.1</td>
<td>12.0 ± 1.2</td>
<td>11.8 ± 1.3</td>
</tr>
<tr>
<td>Socioeconomic status (Hollingshead class)</td>
<td>3.9 ± 1.0</td>
<td>4.0 ± 1.2</td>
<td>3.7 ± 1.1</td>
<td>3.9 ± 1.1</td>
</tr>
<tr>
<td>Total pregnancies</td>
<td>3.7 ± 2.4</td>
<td>3.9 ± 2.5</td>
<td>3.2 ± 1.6</td>
<td>3.6 ± 2.2</td>
</tr>
<tr>
<td>Smoking (cigarettes/d)</td>
<td>5.7 ± 8.5</td>
<td>9.9 ± 8.5</td>
<td>NA</td>
<td>5.5 ± 8.0</td>
</tr>
<tr>
<td>Absolute alcohol intake (mL/d)</td>
<td>30.6 ± 43.7</td>
<td>44.0 ± 56.6</td>
<td>19.1 ± 29.0^2</td>
<td>32.8 ± 47.6</td>
</tr>
<tr>
<td>Selected dietary intakes (oz/d)</td>
<td>1.0 ± 1.5</td>
<td>1.5 ± 1.9</td>
<td>0.6 ± 1.0^2</td>
<td>1.1 ± 1.6</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>72 ± 14</td>
<td>67 ± 15</td>
<td>71 ± 14</td>
<td>69 ± 14</td>
</tr>
<tr>
<td>Carbohydrates (g/d)</td>
<td>259 ± 46</td>
<td>256 ± 46</td>
<td>243 ± 40</td>
<td>250 ± 44</td>
</tr>
<tr>
<td>Fat (g/d)</td>
<td>94 ± 16</td>
<td>89 ± 41</td>
<td>94 ± 43</td>
<td>91 ± 42</td>
</tr>
<tr>
<td>Vitamin B-6 (mg/d)</td>
<td>1.9 ± 0.4</td>
<td>1.8 ± 0.4</td>
<td>1.9 ± 0.3</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Vitamin B-12 (mg/d)</td>
<td>5.5 ± 2.3</td>
<td>5.6 ± 2.1</td>
<td>5.2 ± 1.6</td>
<td>5.4 ± 1.9</td>
</tr>
<tr>
<td>Methionine (g/d)</td>
<td>1.7 ± 0.4</td>
<td>1.5 ± 0.4</td>
<td>1.6 ± 0.4</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>Dietary folate equivalents (mg/d)</td>
<td>466 ± 121</td>
<td>428 ± 129</td>
<td>442 ± 110</td>
<td>434 ± 120</td>
</tr>
<tr>
<td>Food folate (mg/d)</td>
<td>216 ± 83</td>
<td>208 ± 84</td>
<td>224 ± 79</td>
<td>215 ± 82</td>
</tr>
<tr>
<td>Fortified folate (mg/d)</td>
<td>147 ± 61</td>
<td>129 ± 66</td>
<td>128 ± 55</td>
<td>129 ± 61</td>
</tr>
<tr>
<td>Total energy (MJ/d)</td>
<td>9.2 ± 4.4</td>
<td>9.0 ± 3.6</td>
<td>9.0 ± 4.0</td>
<td>9.0 ± 3.7</td>
</tr>
<tr>
<td>Maternal plasma 5-MTHFA (ng/mL)</td>
<td>ND</td>
<td>12.5 ± 7.9</td>
<td>13.5 ± 7.0</td>
<td>13.0 ± 7.5</td>
</tr>
</tbody>
</table>

1 All values are x ± SD. NA, not applicable; ND, not determined; 5-MTHFA, 5-methyltetrahydrofolate.
2 Significantly different from smokers, P ≤ 0.05 (two-tailed independent t test).
3 Values adjusted for energy by the nutrient residual model.

requirements for other clinical and research analyses. In particular, instances of a complete set of unhemolyzed maternal plasma, venous, and arterial cord plasma were limited (n = 15). Matching only the maternal and the venous cord plasma resulted in a larger sample set (n = 58). The maternal age in the present analysis ranged from 17 to 38 y. Maternal characteristics and selected dietary intakes in the present analysis (n = 58) were similar to those in the entire sample (n = 282) (Table 1). The percentage of women smoking during pregnancy reported in the present analysis was 55% (95% CI: 42%, 68%), which was similar to the 48% of the entire sample who reported smoking (95% CI: 42%, 54%).

In the present sample, alcohol intake was higher in the smoking than in the nonsmoking mothers (Table 1). This was also true in the entire sample. In addition, the nonsignificant differences in education, socioeconomic status, and total pregnancies in the present analysis reached significance with the increase in power available in the entire sample. In summary, smokers had lower levels of education (grade completed: 11.5 ± 1.5 compared with 12.0 ± 1.2; P = 0.003), lower socioeconomic status (Hollingshead score: 4.1 ± 1.0 compared with 3.7 ± 1.0; P < 0.001), and a greater number of total pregnancies (4.1 ± 2.7 compared with 3.4 ± 1.0; P = 0.015).

Maternal DFE intakes were estimated at 434 ± 120 µg/d (median: 420 µg/d). This suggests that 79.3% of the subjects had DFE intakes below the Estimated Average Requirement (EAR) of 520 µg/d and 91.4% below the RDA of 600 µg/d for pregnant women. The intake of vitamin B-6 was 1.8 ± 0.4 mg/d (median: 1.8 mg/d) and was below the EAR of 1.6 mg/d in 29.3% of the subjects and below the RDA of 1.9 mg/d in 65.5% of the subjects. In contrast, vitamin B-12 intakes were estimated at 5.4 ± 1.9 µg/d (median: 5.3 µg/d); 96.6% of the subjects met the EAR of 2.2 µg/d and the RDA of 2.6 µg/d.

Maternal and umbilical cord plasma folate

Plasma 5-MTHFA concentrations were higher in both the arterial and venous cord plasma (18.4 ± 7.4 and 18.7 ± 9.6 ng/mL, respectively) than in the maternal plasma (13.5 ± 7.8 ng/mL) (Figure 1). No significant difference between arterial and venous plasma concentrations of 5-MTHFA were observed. In the expanded sample (n = 58), venous cord plasma 5-MTHFA concentrations (16.8 ± 7.5 ng/mL) were again significantly higher than maternal plasma 5-MTHFA concentrations (13.0 ± 7.5 ng/mL) (Figure 1). Exclusion of the 2 subjects determined to be taking folic acid supplements regularly resulted in no changes in the statistical results, although slight reductions were found in the mean and SDs for both the maternal-arterial-venous analysis (n = 14) and the maternal-venous analysis (n = 56).

Effect of smoking

Cigarettes smoked by the mothers at the first prenatal visit as compared with other quantitative smoking estimates had the strongest Pearson’s correlation with plasma 5-MTHFA determinations. Venous cord plasma 5-MTHFA was positively associated with maternal plasma 5-MTHFA (r = 0.60, P < 0.001; Figure 2) and negatively associated with cigarettes smoked by the mothers at the first prenatal visit (r = −0.31, P = 0.019).
Cigarettes smoked by the mothers was not significantly associated with maternal plasma 5-MTHFA ($r = -0.05, P = 0.71$). Similarly, prepregnancy cigarettes smoked was negatively associated with venous cord 5-MTHFA ($r = -0.30, P = 0.023$) and not associated with maternal 5-MTHFA ($r = -0.04, P = 0.78$). Prepregnancy and first prenatal visit cigarettes smoked were strongly correlated ($r = 0.99, P < 0.001$). Cigarettes smoked by the fathers (data available for 53 maternal-cord pairings) was significantly correlated only with maternal first prenatal visit plasma 5-MTHFA concentrations were similar to our previous finding at 24 wk of gestation ($r = -0.21, P = 0.043; n = 92$) (1). Exclusion of the 2 subjects who were taking folic acid supplements had no statistical effect on these bivariate associations.

Venous cord plasma 5-MTHFA concentrations in smoking mothers ($15.1 \pm 7.6 \text{ ng/mL}; n = 32$) were significantly lower than venous cord concentrations in nonsmoking mothers ($19.0 \pm 7.0 \text{ ng/mL}; n = 26$) (Figure 3). These differences persisted after the inclusion of maternal plasma 5-MTHFA concentrations ($P = 0.039$), energy-adjusted DFE intakes ($P = 0.038$), and both maternal plasma 5-MTHFA and adjusted DFE ($P = 0.034$) in general linear models as covariates. Maternal plasma 5-MTHFA concentrations were slightly but not significantly lower in the smoking ($12.5 \pm 7.9 \text{ ng/mL}; n = 32$) than in the nonsmoking ($13.5 \pm 7.0 \text{ ng/mL}; n = 26$; $t_{58} = 0.50, P = 0.62$) mothers (Figure 3), and the inclusion of maternal energy-adjusted DFE intake as a covariate had no effect.

Maternal plasma 5-MTHFA was positively associated (standardized $\beta = 0.59, P < 0.001$) and cigarettes smoked by the mothers at the first prenatal visit was negatively associated (standardized $\beta = -0.28, P = 0.008$) with venous cord plasma 5-MTHFA concentrations and were the only independent variables remaining in the parsimonious multiple linear regression model (Table 2). These associations also remained significant in a controlled model that included energy intake, maternal alcohol consumption, education, maternal age, and BMI. Energy-adjusted DFE intakes and socioeconomic status were also considered but excluded from the model because of collinearity with other independent variables. In the present sample for which maternal and umbilical cord concentrations were measured ($n = 58$), infant birth weight did not differ significantly between smoking and nonsmoking mothers ($3190 \pm 107$ and $3345 \pm 119$, respectively; $P = 0.34$) in a simple univariate analysis; however, the effect of maternal smoking on birth weight was significant when gestational age and infant sex were entered as covariates ($P = 0.042$).

**DISCUSSION**

Smoking by inner-city African American women during pregnancy significantly decreases 5-MTHFA concentrations in cord plasma. This effect is independent of maternal folate intake and independent of the effect of maternal smoking on maternal plasma 5-MTHFA concentrations. Venous cord plasma 5-MTHFA was

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**FIGURE 1.** Mean (±SD) 5-methyltetrahydrofolic acid concentrations in maternal plasma and arterial and venous cord plasma samples from African American women. Significantly different from arterial and venous cord plasma, by Tukey’s honestly significantly different test ($P < 0.01$), after significance was determined by repeated-measures ANOVA ($P = 0.0023$). †Significantly different from venous cord plasma, $P < 0.0001$ (paired $t$ test).

**FIGURE 2.** Scatterplot of the relation between maternal and venous cord plasma 5-methyltetrahydrofolic acid concentrations in African American women ($n = 58$). Each circle represents an individual data point. Bivariate associations were determined by Pearson’s correlations: $r = 0.60, R^2 = 0.36$, $P < 0.001$. $y = 0.60x + 2.957$.

**FIGURE 3.** Mean (±SD) venous cord and maternal plasma 5-methyltetrahydrofolic acid concentrations in smoking African American women. *Significantly different from the nonsmoking group, $P = 0.0498$ (independent $t$ test).
TABLE 2
Results of multiple linear regression with venous cord plasma
5-methyltetrahydrofolate (5-MTHFA) concentrations as the dependent
variable and dietary folate, smoking, drinking, and maternal plasma
5-MTHFA as independent variables

<table>
<thead>
<tr>
<th>Group and model variables</th>
<th>Model $R^2$</th>
<th>Standardized β</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parsimonious model</td>
<td>0.44</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Maternal plasma</td>
<td>—</td>
<td>0.59</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5-MTHFA (ng/mL)</td>
<td>—</td>
<td>—</td>
<td>0.008</td>
</tr>
<tr>
<td>Cigarettes smoked per day</td>
<td>—</td>
<td>−0.28</td>
<td>0.001</td>
</tr>
<tr>
<td>Controlled model</td>
<td>0.47</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Maternal plasma</td>
<td>—</td>
<td>0.59</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5-MTHFA (ng/mL)</td>
<td>—</td>
<td>—</td>
<td>0.009</td>
</tr>
<tr>
<td>Cigarettes smoked per day</td>
<td>—</td>
<td>−0.31</td>
<td>0.53</td>
</tr>
<tr>
<td>Dietary vitamin B-12 (µg)</td>
<td>—</td>
<td>−0.07</td>
<td>0.75</td>
</tr>
<tr>
<td>Drinking (absolute oz of alcohol)</td>
<td>—</td>
<td>0.04</td>
<td>0.60</td>
</tr>
<tr>
<td>Education (y)</td>
<td>—</td>
<td>0.06</td>
<td>0.31</td>
</tr>
<tr>
<td>Mother’s age (y)</td>
<td>—</td>
<td>0.11</td>
<td>0.37</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>—</td>
<td>−0.10</td>
<td>0.37</td>
</tr>
</tbody>
</table>

1 Independent variables in the multiple linear regression models were
included on the basis of biological plausibility as reported previously in the
literature, the influence on the model $R^2$ value, and the degree of collinearity
with other independent variables ($n = 58$). A backward stepwise parsimo-
nious model with all included independent variables with $P$ values < 0.10 and
a controlled model with potential confounders included as independent vari-
ables were generated.

20.5% lower in the infants from smoking mothers compared with infants from nonsmoking mothers.

This is the first study to report an effect of maternal smoking on infant folate status. Previously in the United Kingdom, Relton et al (14) showed that neonatal erythrocyte folate was associated with maternal erythrocyte folate and maternal vitamin B-12 and that smoking decreased maternal erythrocyte folate ($n = 319$) but not neonatal erythrocyte folate ($n = 271$). However, smoking was assessed categorically (never, ever, or current), and mother-infant paired data were used only for maternal-neonatal vitamin correlations and not for the effects of smoking on neonates. Bjørke Monsen et al (20) reported no differences in serum folate and whole-blood folate between Norwegian neonates from smoking ($n = 30$) and nonsmoking mothers ($n = 143$) at birth and at 6 wk. A slight increase in serum folate and a significant decrease in serum vitamin B-12 concentrations were observed in neonates of “heavy” smoking mothers defined in that study as smoking >7 cigarettes/d.

The present study had several limitations. Erythrocyte folate, total folate, and forms of folate other than 5-MTHFA were not assessed in the present study. Vitamin B-12 measurements were also not completed because of limited volumes of cord blood samples and other analytic priorities. In addition, accurate determinations of maternal smoking and folate intake are difficult and subject to error, as discussed previously (1). However, accurate and specific determinations of plasma 5-MTHFA were made by HPLC-MS-ESI for maternal-infant pairings, and prepregnancy smoking, smoking at the time of the first prenatal visit, and the father’s smoking habits were assessed both quantitatively (cigarettes smoked/d) and categorically (smokers compared with nonsmokers).

The present HPLC-MS-ESI method can be used to determine other forms of folate, including 5-MTHFA, tetrahydrofolate, 5-formyltetrahydrofolate, and folic acid if appropriate internal standards are available, as done recently in erythrocytes (40) but not in the present study. An effect of smoking on 5-MTHFA would likely persist for total plasma folate because 5-MTHFA is estimated to consist of >90% of total plasma folate in persons with low folate, such as the present population sample, and remain >80% in persons with higher folate concentrations (41). Little data on the concentrations of specific forms of folate in cord and infant plasma and erythrocytes are available, and the placental transport of the individual forms of folate has not been fully elucidated. Folic acid transport is inhibited in the presence of 5-MTHFA in BeWo monolayers (42).

Maternal plasma 5-MTHFA concentrations in the present study (13.0 ± 7.5 ng/mL) were lower than those previously published in the same population of women at 24 wk of gestation (18.0 ± 7.1 ng/mL) (1). A decrease was expected because of the normal physiologic blood and plasma volume expansion that occurs during pregnancy (43). However, the increase in maternal plasma volume from 24 wk gestation to the time of infant delivery in this population was previously estimated at 10% (44), which would account for a difference of ≈1.8 ng/mL. The lower maternal plasma 5-MTHFA concentrations at delivery may also have been a consequence of reduced DFEs as pregnancy progressed, because the mean, median, and SD for DFE intakes were all lower in the present sample (mean: 434; median: 420; SD: 120 µg/d) than in previous samples at 24 wk gestation (mean: 605 µg/d; median: 590 µg/d; SD: 181 µg/d). Fortified folate intake was also estimated to be 147 ± 61 µg/d in the entire sample at delivery ($n = 282$) and 129 ± 61 in the present sample ($n = 58$), as compared with 198 ± 98 µg/d determined previously at 24 wk gestation (1). Gustatory sensitivity decreases during pregnancy (44), and, in the present population, the mass intakes of protein, carbohydrates, and fat are lower at delivery than at 24 wk gestation (32). In addition, folate catabolism progressively increases during pregnancy (45), and folate absorption and utilization may increase significantly after 27 wk gestation (46).

Folate fortification appears to have resulted in dietary folate increases greater than the anticipated 70–130 µg/d (2). Folate values in the US Department of Agriculture National Nutrient Database for Standard Reference, release 14, are based on enrichment specifications (35). Therefore, the estimates of fortified folate intake in this population suggest that fortified food products were eaten in greater quantities than those estimated at the time of fortification. In addition, the folate intakes in the present study may be underestimates of actual intakes because folate fortification of the food supply in excess of enrichment targets has been reported (47).

Cord plasma 5-MTHFA concentrations in the present study (16.8 ± 7.5 ng/mL) were significantly higher than maternal plasma 5-MTHFA concentrations (13.0 ± 7.5 ng/mL) but were similar to plasma folate concentrations measured in an Irish population by Molloy et al (24): 20.7 ng/mL in cord plasma and 11.3 ng/mL in maternal plasma. Thus, the present study supports concentative maternal-fetal folate transport via the placenta. Cigarettes smoked by the mothers and fathers was not associated with altered maternal plasma 5-MTHFA, and maternal plasma 5-MTHFA was not significantly different between smokers and nonsmokers. This was likely due to restricted statistical power (∼10–20%) because of the small number of subjects. Smoking and smoke exposure have been negatively associated with serum
and erythrocyte folate (14, 18) and plasma 5-MTHFA (1) in larger sample sizes. There was sufficient power (~50%) in the present study to determine an effect of maternal smoking on venous cord 5-MTHFA.

Maternal smoking at the first prenatal visit (17.0 ± 6.8 wk gestation) appeared to affect cord plasma 5-MTHFA concentrations to a greater extent than maternal smoking affected maternal folate status at the time of delivery. However, maternal folate status is the major determinant of infant status, because maternal 5-MTHFA concentrations accounted for 35% of the variation and cigarette smoking accounted for 10% of the variation in venous cord 5-MTHFA. The effect of decreased cord folate concentrations at the time of delivery on neural tube defects is difficult to assess given the importance of adequate folate status at the time of conception and neural tube formation. It is possible that folate transport may be affected by smoking during early embryogenesis, because it has been speculated that smoking may impair folate receptor activity and the expression in squamous cell cancers of the lung (48). Further research on the effect of smoking on folate transport across the placenta and in other cell systems is required.

Extrapolation of the present findings to other populations requires caution given the low prevalence of methylenetetrahydrofolate reductase mutations, low DFE intakes, and adequate vitamin B-12 intakes in African Americans. The present study supports the hypothesis that lifestyle choices, such as smoking during pregnancy, may be partly responsible for the smaller reductions in neural tube defects than those expected after folate acid fortification in selected populations, such as inner-city African Americans.

All authors contributed to the study design and concept and to the revision of the manuscript. JHH and NS were coprincipal investigators. JHH and RJS coordinated and supervised participant recruitment and sample collection. KDS and RJP performed the 5-methyltetrahydrofolate measurements. KDS conducted the statistical analyses and wrote the first draft of the manuscript. None of the authors had a conflict of interest.

REFERENCES

n−3 Fatty acids are positively associated with peak bone mineral density and bone accrual in healthy men: the NO2 Study

Magnus Högström, Peter Nordström, and Anna Nordström

ABSTRACT

Background: Knowledge of the influence of nutritional intake on bone health is limited. Polyunsaturated fatty acids have been suggested to influence bone growth and modeling in humans, although data are sparse.

Objective: The objective was to investigate the role of fatty acids in bone accumulation and the attainment of peak bone mass in young men.

Design: The cohort studied consisted of 78 healthy young men with a mean age of 16.7 y at baseline. Bone mineral density (BMD; in g/cm²) of total body, hip, and spine was measured at baseline and at 22 and 24 y of age. Fatty acid concentrations were measured in the phospholipid fraction in serum at 22 y of age.

Results: Concentrations of n−3 fatty acids were positively associated with total BMD (r = 0.27, P = 0.02) and spine BMD (r = 0.25, P = 0.02) at 22 y of age. A positive correlation between n−3 fatty acid concentrations and the changes in BMD at the spine (r = 0.26, P = 0.02) was found between 16 and 22 y of age. Concentrations of docosahexaenoic acid (DHA, 22:6n−3) were positively associated with total BMD (r = 0.32, P = 0.004) and BMD at the spine (r = 0.30, P = 0.008) at 22 y of age. A positive correlation was also found between DHA concentrations and the changes in BMD at the spine (r = 0.26, P = 0.02) between 16 and 22 y of age.

Conclusion: The results showed that n−3 fatty acids, especially DHA, are positively associated with bone mineral accrual and, thus, with peak BMD in young men. Am J Clin Nutr 2007;85:803–7.

KEY WORDS: Peak bone mineral density, bone accrual, men, docosahexaenoic acid, n−3 fatty acids

INTRODUCTION

Osteoporosis and related fractures are increasing causes of mortality and painful physical impairment in the elderly, especially in the Western world (1, 2). Bone mineral accrual during childhood and adolescence is thought to play a vital role in preventing osteoporosis (3–5). Identifying and optimizing factors influencing peak bone mass is thus important for the prevention of osteoporosis and related fractures.

Genetic factors, estimated to be responsible for ≈70% of the variance in bone mass (6, 7), cannot be influenced; other factors affecting bone, such as nutritional intake, physical activity, and body mass index (BMI), can be influenced, thereby decreasing the risk of osteoporosis and its consequences (8).

Knowledge of the influence of nutritional intake on bone health is limited. Estimated intakes of polyunsaturated fatty acids (PUFAs) and saturated fatty acids have been suggested to influence bone growth and modeling in humans, although data are sparse (9, 10). A pilot study suggested that dietary supplementation with evening primrose oil rich in γ-linolenic acid (GLA) and fish oil rich in eicosapentaenoic acid (EPA) may decrease bone turnover and increase bone mineral density (BMD) in elderly patients (11).

Animal studies have shown that a dietary intake of long-chain n−3 PUFAs may influence both bone formation and bone resorption (12, 13). Fatty acids have also been reported to have an influence on bone metabolism, including an increase in periosteal bone formation in animal studies (14, 15).

No study to date has investigated the association between concentrations of different fatty acids measured in serum and BMD in men. Therefore, the aim of this 8-y prospective and retrospective study was to investigate a possible role of fatty acids in bone accumulation and the attainment of peak bone mass in young postpubertal men.

SUBJECTS AND METHODS

Subjects

Beginning in 1994, we recruited 95 healthy adolescent white males from high schools and athletic clubs in Umeå in northern Sweden for this longitudinal study—the Northern Osteoporosis and Obesity Study (NO2 Study). The initial aim was to investigate factors important for the development of BMD (16) and body-composition variables such as fat mass and lean body mass (17, 18). After a mean period of 5 y and 11 mo, 84 of the original participants agreed to participate in a first follow-up examination. At this follow-up, blood samples were obtained. One of the participants received a diagnosis of anorexia nervosa and depression between baseline and the follow-up and was therefore excluded, and 5 subjects were excluded for declining to leave blood samples, which left 78 participants. Seventy-three of these men

participated in the second follow-up conducted after a mean of 7 y and 11 mo.

Questionnaire on lifestyle

Physical activity was measured by questionnaire and defined as the self-reported mean activity associated with sweating or breathlessness each week during the last year. The questionnaire has been used since the data collection began in 1995 (18) and at all follow-ups. In subjects with organized physical training in local sports clubs, the self-reported activity of each subject was also validated by the team coach at the start of this study. The group’s physical activity consisted mainly of playing ice hockey, soccer and floor ball, distance running, and some weight training. The questionnaire used at baseline and at the follow-ups also included questions on smoking habits, known illnesses, and medication use. Pubertal stage according to Tanner was determined via self-examination of pubic hair and questions on growth of beard and height development at baseline. None of the participants had any disease or were taking any medication known to affect bone metabolism.

All data were collected at the Sports Medicine Unit at the University Hospital of Northern Sweden. All of the participants gave informed consent, and the Ethics Committee of the Medical Faculty, Umeå University, Umeå, Sweden, approved the study protocol.

Anthropometric measurements

Weight and height were measured in light clothing. Weight was measured to the nearest 0.1 kg by use of a digital scale, and height was measured to the nearest 0.5 cm against a wall-mounted stadiometer.

Bone mineral density measurements

The BMD (g/cm²) of the total body and right hip was measured by using a Lunar DPX-L (Lunar Co, Waukesha, WI) dual-energy X-ray absorptiometer with version 4.6e software. The CV (CV, ie, SD/mean) was determined by scanning one person 7 times on the same day, with repositioning between each scan. Accordingly, the CVs were 0.7% with the total body scan and ≈1% for the total hip scan. The BMD of the spine was derived from the total body scan. One investigator (AN) performed all the analyses. To maximize precision, the scaling option was used and set to 200. To evaluate the region-of-interest program, 2 different persons were scanned. The first person was scanned 7 times on the same day, with repositioning between each scan. The CV was then calculated to be 1.1% for spine BMD. The second person was scanned on different days for a total of 10 times. The CV then increased to 2.5%. The equipment was calibrated each day by using a standardized phantom to detect drifts in BMD measurement. All scans were made by using the same Lunar DPX-L.

Fatty acid profile in serum phospholipids

 Serum was obtained under a nonfasting condition from 78 men during the first follow-up, ie, at 22 y of age. Total plasma lipids were extracted according to Folch et al (19), and the phospholipids were isolated on 400 mg aminopropyl solid-phase extraction columns according to Helland et al (20). Phospholipids were eluted from the columns with methanol, evaporated with hot nitrogen, and transmethyalted with fresh sodium methoxide. Fatty acid methyl esters were extracted into hexane containing 20 mg butylated hydroxyl toluene (BHT)/L as an antioxidant and separated on a 100 m × 0.25 mm (internal diameter) capillary gas chromatography column (SP-2256; Supelco, Bellefonte, PA), with hydrogen as the carrier gas and flame ionization detection. The results are expressed as grams fatty acids/ g serum phospholipids.

Statistical analyses

All data are presented as means ± SDs. Bivariate correlations were measured by using Pearson’s correlation coefficients (r). The independent contributions of fatty acids to BMD at each site were investigated by using linear regression, including weight, height, and physical activity as independent variables. Differences between 3 groups were tested by using analysis of variance (ANOVA) with Bonferroni’s test for post hoc comparisons. The SPSS software for personal computers (version 11.5; SPSS Inc, Chicago, IL) was used for the statistical analyses. P values <0.05 was considered statistically significant.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n = 78)</th>
<th>First follow-up (n = 78)</th>
<th>Second follow-up (n = 73)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>16.7 ± 0.5&lt;sup&gt;2&lt;/sup&gt;</td>
<td>22.6 ± 0.7&lt;sup&gt;1&lt;/sup&gt;</td>
<td>24.6 ± 0.6</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.8 ± 9.1&lt;sup&gt;2&lt;/sup&gt;</td>
<td>81.7 ± 11.1</td>
<td>83.0 ± 10.8</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>179 ± 6&lt;sup&gt;6&lt;/sup&gt;</td>
<td>181 ± 6</td>
<td>181 ± 6</td>
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<tr>
<td>Physical activity (h/wk)</td>
<td>6.9 ± 4.2&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4.8 ± 3.8</td>
<td>3.6 ± 2.9</td>
</tr>
<tr>
<td>BMD (g/cm²)</td>
<td></td>
<td></td>
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<tr>
<td>Total body</td>
<td>1.22 ± 0.08&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.31 ± 0.07</td>
<td>1.31 ± 0.7</td>
</tr>
<tr>
<td>Total hip</td>
<td>1.27 ± 0.13</td>
<td>1.26 ± 0.15</td>
<td>1.23 ± 0.15</td>
</tr>
<tr>
<td>Spine</td>
<td>1.16 ± 0.11&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.27 ± 0.12</td>
<td>1.30 ± 0.14</td>
</tr>
<tr>
<td>Fatty acid profile (g/100 g serum phospholipids)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>—</td>
<td>29.1 ± 1.2</td>
<td>—</td>
</tr>
<tr>
<td>Palmitoleic acid (16:1)</td>
<td>—</td>
<td>0.8 ± 0.4</td>
<td>—</td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>—</td>
<td>15.6 ± 1.4</td>
<td>—</td>
</tr>
<tr>
<td>Oleic acid (18:1)</td>
<td>—</td>
<td>11.9 ± 1.9</td>
<td>—</td>
</tr>
<tr>
<td>Linoleic acid (18:2n−6)</td>
<td>—</td>
<td>22.9 ± 3.0</td>
<td>—</td>
</tr>
<tr>
<td>Eicosatrienoic acid</td>
<td>—</td>
<td>3.7 ± 0.8</td>
<td>—</td>
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<tr>
<td>(20:3n−6)</td>
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<tr>
<td>Arachidonic acid (20:4n−6)</td>
<td>—</td>
<td>8.7 ± 1.4</td>
<td>—</td>
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<tr>
<td>Eicosapentaenoic acid (20:5n−3)</td>
<td>—</td>
<td>1.8 ± 1.0</td>
<td>—</td>
</tr>
<tr>
<td>Docosapentaenoic acid (22:5n−3)</td>
<td>—</td>
<td>1.3 ± 0.3</td>
<td>—</td>
</tr>
<tr>
<td>Docosahexaenoic acid (22:6n−3)</td>
<td>—</td>
<td>3.6 ± 1.0</td>
<td>—</td>
</tr>
<tr>
<td>PUFU</td>
<td>—</td>
<td>42.5 ± 2.1</td>
<td>—</td>
</tr>
<tr>
<td>MUFA</td>
<td>—</td>
<td>12.7 ± 1.9</td>
<td>—</td>
</tr>
<tr>
<td>SFA</td>
<td>—</td>
<td>44.7 ± 1.5</td>
<td>—</td>
</tr>
<tr>
<td>n−6</td>
<td>—</td>
<td>35.6 ± 2.3</td>
<td>—</td>
</tr>
<tr>
<td>n−3</td>
<td>—</td>
<td>7.0 ± 1.9</td>
<td>—</td>
</tr>
<tr>
<td>n−6n−3</td>
<td>—</td>
<td>5.4 ± 1.3</td>
<td>—</td>
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</tbody>
</table>

<sup>1</sup> All values are x ± SD. PUFU, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid.

<sup>2</sup> Significantly different from first and second follow-ups, P < 0.01.

<sup>3</sup> Significantly different from second follow-up, P < 0.01.

<sup>4</sup> Significantly different from first follow-up, P < 0.05.
RESULTS

The participants’ fatty acid profile, age, weight, height, physical activity, and BMD of the total body, spine, and hip at baseline and follow-up visits are presented in Table 1. The concentration of α-linolenic acid could only be detected in ≈50% of the subjects; therefore, these data are not presented.

Bivariate correlations between the different fatty acids and BMD measured at 22 y of age and the changes in BMD (ΔBMD) between 16 and 22 y and 22 and 24 y of age are presented in Table 2. In summary, BMD of the total body measured at 22 y of age showed a significant negative correlation with serum concentrations of oleic acid \((r = -0.26, P = 0.02)\) and monounsaturated fatty acids \((r = -0.25, P = 0.02)\) and a significant positive correlation with docosahexaenoic acid (DHA) \((r = 0.32, P = 0.004, \text{Figure 1})\) and n–3 fatty acids \((r = 0.27, P = 0.02)\). BMD of the spine measured at 22 y of age showed a positive association with DHA \((r = 0.30, P = 0.008)\) and n–3 fatty acids \((r = 0.25, P = 0.02)\). ΔBMD of the spine between 16 and 22 y of age showed a positive association with arachidonic acid \((r = 0.25, P = 0.02)\), DHA \((r = 0.26, P = 0.02)\), and n–3 fatty acids \((r = 0.26, P = 0.02)\) and a negative association with the ratio of n–6 to n–3 fatty acids \((r = -0.26, P = 0.02)\).

The independent relations between the different fatty acids and BMD were also evaluated by using linear regression and including weight, height, and physical activity as explanatory variables. BMD of the total body then showed an independent relation with eicosapentaenoic acid (EPA) \((\beta = 0.31, P = 0.02)\), DHA \((\beta = 0.31, P = 0.02)\), and n–3 fatty acids \((\beta = 0.35, P = 0.007)\) at 22 y of age (data not shown). BMD of the spine measured at 22 y of age showed an independent significant relation with DHA \((\beta = 0.28, P = 0.03)\) and n–3 fatty acids \((\beta = 0.31, P = 0.01)\) (data not shown).

The independent association between concentrations of fatty acids and the changes in BMD between 16 and 22 y of age and between 22 and 24 y were also investigated. The concentrations of different fatty acids were used as explanatory variables together with changes in weight, height, and physical activity during the same period. ΔBMD of the spine between 16 and 22 y of age was then found to be independently related to DHA \((\beta = 0.28, P = 0.03)\) and n–3 fatty acids \((\beta = 0.28, P = 0.04)\) measured at 22 y of age. Finally, ΔBMD of the spine between 22 and 24 y of age was independently related to concentrations of palmitoleic acid measured at 22 y of age \((\beta = 0.27, P = 0.03)\).
DISCUSSION

The novelty of our study was in the measurement of fatty acids in the serum phospholipid fraction in healthy men and their association with BMD and bone accrual in our cohort. Our key finding was a positive association between n−3 fatty acids and BMD of the total body and spine and the accumulation of BMD at the spine between 16 and 22 y of age in this cohort of healthy young men.

To our knowledge, studies performed in humans that have investigated the possible role of n−3 fatty acids in skeletal health are sparse, and the few that do exist are supplement studies that did not investigate the concentrations of PUFAs in serum. The data from supplement studies in the elderly suggest that n−3 fatty acid intakes may promote BMD maintenance in this population. In one study, elderly postmenopausal women were given a mixture of fish oil and evening primrose oil with a high content of EPA and γ-linolenic acid (GLA), whereas the control group received coconut oil (11). After 18 mo, both groups had lower concentrations of bone turnover markers such as osteocalcin and deoxypyridinoline. In the group treated with GLA and EPA, BMD at the lumbar spine was maintained and BMD of the femoral neck even increased. In the control group, BMD decreased at both sites (11). However, it cannot be concluded from this study whether n−3 or n−6 fatty acids or both are beneficial for BMD. In another study, 20 calcium nephrolithiasis patients were given a fish-oil supplement. The results included lower plasma prostaglandin E2 concentrations, serum calcitriol concentrations, and markers of bone resorption, whereas calcium resorption increased and calcium excretion decreased (21). Thus, it seems that n−3 fatty acids might maintain bone density in the elderly, although the mechanism is not known, and the evidence thus far is sparse at best. This was also confirmed in a large cohort study in which food intakes of PUFAs were investigated in 1532 men and women aged 45–90 y. The ratio of n−6 to n−3 fatty acids showed a negative association with BMD of the hip in both men and women (22). The effects of n−3 fatty acids on bone health may be multifaceted. Postulated mechanisms include effects on calcium absorption in the intestine, reductions in bone resorption because of lower urinary excretion of calcium, and enhanced synthesis of bone collagen (23–25). n−3 Fatty acids have also been put forward as likely candidate inhibitors of the production of cytokines such as interleukin 6, interleukin 1, and tumor necrosis factor, which are implicated in the pathogenesis of osteoporosis (26–31).

No studies of the possible role of n−3 fatty acids in bone mineral accrual in growing humans have been conducted. However, animal studies suggest that n−3 fatty acids may have a positive role in this process. Iwami-Morimoto et al investigated the influence of fish-oil supplementation (rich in n−3 fatty acids) compared with that of corn oil (rich in n−6 fatty acids) on experimental tooth development in rats. n−3 Fatty acid supplementation decreased the number of osteoclasts by 60% and alveolar bone resorption by 80% (13). Reinwald et al (32) found that rats with n−3 deficient bone tissue had a high ratio of n−6 to n−3 fatty acids and diminished bone strength. Repletion with dietary n−3 fatty acids restored the ratio of n−6 to n−3 fatty acids in bone compartments and reversed compromised bone modeling. In contrast, Claassen et al (25) found that growing rats supplemented with increasing ratios of GLA to EPA (ie, n−6 to n−3 fatty acids) had lower concentrations of bone resorption markers and higher bone calcium contents than did control subjects supplemented with linoleic acid and α-linolenic acid.

The association between n−3 fatty acids and total and spine BMD in our study seemed to mostly be due to the positive correlation of a specific n−3 fatty acid, namely DHA, with both BMD at 22 y of age and changes in BMD of the spine between 16 and 22 y. No studies have investigated the association between serum concentrations of individual PUFAs and bone density in humans. However, experimental animal studies of supplementation support our findings that DHA is positively related to bone density and bone accrual. Kruger and Schollum (33) found that DHA concentrations in red blood cell membranes were associated with BMD, and with calcium absorption in bone, in a cohort of growing rats fed a semisynthetic diet supplemented with tuna oil. Others have found that DHA increases intestinal calcium ATPase activity and may affect calcium absorption through this mechanism (34). Weiler and Fitzpatrick-Wong (35) found that higher concentrations of plasma DHA were associated with lessened bone resorption in piglets fed diets containing different ratios of n−6 to n−3 fatty acids. Thus, other experimental studies support our findings and clearly indicate a possible relation between dietary supplementation with DHA and various bone variables.

The present study had some limitations. The cohort studied was not randomly selected from the general population, but rather consisted of volunteers from high schools and sports clubs. Therefore, inferences with respect to the general population should be made with caution. No information on dietary patterns was available, which may have been useful to further strengthen the relation between fatty acids and BMD. We found relations between changes in BMD and both palmitoleic acid and arachidonic acid. We have no certain theoretical explanation for these findings. In this context it should be noted that, because many correlations were tested, it is more than likely that some of the significant correlations found may have been due to type I errors.

In conclusion, this was the first study to investigate the association between individual PUFAs, BMD, and bone mineral accrual. In a cohort of healthy young men, we found that concentrations of n−3 fatty acids, especially DHA, were positively associated with peak BMD in the total body and spine and with bone accrual in the spine. More studies are needed to confirm our results and investigate the relation between individual PUFAs and BMD further.

AN and PN collected the material. AN and MH collected background information and drafted the first version of manuscript. All authors worked on the manuscript and approved the final version. None of the authors had any conflicts of interest.

REFERENCES

Altered postprandial glucose, insulin, leptin, and ghrelin in liver cirrhosis: correlations with energy intake and resting energy expenditure

Evangelos Kalaitzakis, Ingyar Bosaeus, Lena Öhman, and Einar Björnsson

ABSTRACT

Background: Liver cirrhosis is associated with reduced energy intake and increased resting energy expenditure.

Objective: We aimed to investigate the possible role of glucose, insulin, leptin, and ghrelin in the pathogenesis of these alterations.

Design: Nutritional status, energy intake, resting energy expenditure, and fasting glucose, insulin, and leptin were measured in 31 patients with cirrhosis. Postprandial glucose, insulin, C-peptide, leptin, and ghrelin responses were measured in a subgroup of patients after a standard meal. Ten healthy subjects served as controls.

Results: Patients with cirrhosis had a lower energy intake (P < 0.05), higher resting energy expenditure (P < 0.05), higher fasting leptin (P < 0.05), and higher insulin resistance (P < 0.001) than did the healthy controls. In the patients with cirrhosis, fasting leptin was negatively correlated with resting energy expenditure (r = −0.38, P < 0.05) but not with energy intake. In control subjects, leptin was negatively correlated with energy intake (r = −0.72, P < 0.05) but not with resting energy expenditure. The patients with cirrhosis had higher postprandial glucose (P < 0.001) and lower ghrelin (P < 0.05) concentrations at 4 h postprandially than did the control subjects. The increase in ghrelin from its minimal postmeal value to 4 h postmeal was negatively correlated (r = −0.66, P = 0.014) with weight loss in the patients with cirrhosis. Energy intake was negatively correlated (r = −0.42, P < 0.01) with the postprandial increase in glucose.

Conclusions: In cirrhosis, altered postprandial glucose and ghrelin are associated with reduced energy intake and weight loss, respectively, and the effects of leptin on energy intake and expenditure seem to be altered. Insulin resistance might be involved in these altered postprandial responses. Am J Clin Nutr 2007;85:808–15.

KEY WORDS Glucose, insulin, leptin, ghrelin, insulin resistance, energy intake, resting energy expenditure, liver cirrhosis, malnutrition

INTRODUCTION

Malnutrition is common in patients with liver cirrhosis, with a reported prevalence as high as 80% depending on the severity of liver disease (1–3). The mechanisms of malnutrition in cirrhosis are not completely understood. Both poor dietary intake (3–5) and increased basal energy expenditure have been reported to contribute to a negative energy balance in patients with cirrhosis (1, 6–11).

Insulin resistance is common in patients with cirrhosis (3, 7, 12) and is possibly associated with impairment of nutritional status (12). An elevated postprandial insulin concentration has been proposed as a factor that induces satiety and a subsequent reduction in energy intake in liver cirrhosis (12). However, the relation of postprandial hyperglycemia to energy intake, which has been shown to occur in cirrhosis (12), is unexplored in this group of patients.

Leptin and ghrelin are known to influence energy expenditure and energy intake in humans (13). Leptin circulates in free and bound form, and it has been shown to suppress energy intake and stimulate energy expenditure, whereas ghrelin has been shown to rise before a meal thus enhancing appetite and food intake (13). The basal concentrations of leptin and ghrelin have been reported to be deranged in liver cirrhosis (5, 9, 14–19), but only few studies are available on the relations of leptin and ghrelin to energy intake and resting energy expenditure (REE) in these patients (5, 18, 19). In a previous report, no correlation was found between total leptin concentration and REE in patients with cirrhosis with adequate food intake (18). Also, bound (but not free) leptin was shown to be increased and positively correlated with REE in patients with postviral cirrhosis on a weight-maintaining diet (9). To our knowledge, the relation of leptin to spontaneous energy intake and REE in patients with cirrhosis of various etiologies has not been previously investigated. Also, data are lacking on postprandial changes in leptin and ghrelin in patients with cirrhosis.

Insulin has been reported to be essential for meal-induced ghrelin suppression (20–22) and to acutely increase leptin in healthy persons (23). An inverse relation between leptin and ghrelin has been observed, and it has been proposed that leptin could be of importance for suppression of basal ghrelin in normoinsulinemic subjects (24). Thus, to study the potential importance of these hormones for energy intake and REE, they need to...
be investigated together, a study not previously undertaken in liver cirrhosis.

The main aim of the current study was to investigate the relation of basal and postprandial concentrations of plasma glucose, insulin, leptin, and ghrelin to energy intake and REE. A secondary aim was to study the interrelations of postprandial plasma glucose, insulin, leptin, and ghrelin in patients with cirrhosis.

SUBJECTS AND METHODS

Thirty-one consecutive patients with liver cirrhosis attending the outpatient clinic of the Department of Internal Medicine at Sahlgrenska University Hospital, Gothenburg, Sweden, were enrolled in the study. The diagnosis of liver cirrhosis was established histologically; on the basis of its clinical, laboratory, endoscopic, or imaging features; or both. The severity of liver disease was assessed according to the Child-Pugh and the Model for End Stage Liver Disease scores (25). Patients with malignancy, infections, known gastrointestinal or renal disease, significant respiratory or cardiac dysfunction, insulin-dependent diabetes mellitus, hepatorenal syndrome, untreated thyroid dysfunction, and hepatic encephalopathy grade II–IV were excluded. Patients with alcoholic cirrhosis had been abstinent for ≥6 mo at inclusion. All had normal serum creatinine and had undergone gastroscopy in the previous 6 mo. Twenty-six of the 31 patients had endoscopic evidence of esophageal varices, and 20 of the 31 had evidence of portal hypertensive gastropathy. None of the patients had macroscopic evidence of gastric mucosal atrophy. Two patients were found to have diabetes mellitus on blood sampling for purposes of this study. Six patients had mild ascites detectable by ultrasonography at inclusion and were treated with spironolactone. None had peripheral edema. Ten age-, sex-, and body mass index (BMI)–matched healthy weight-stable volunteers, mainly health-care professionals, acted as controls. Most of them had participated in several studies as healthy volunteers before, none was taking any medications, none was obese, all denied alcohol overconsumption, and all had normal liver function tests. The study was approved by the ethics committee of the University of Gothenburg and informed consent was obtained from all subjects.

Assessment of nutritional status

Weight was measured without shoes and in light clothing. Of 6 patients with mild ascites, every effort was made to calculate dry weight, which is defined as body weight after taking into consideration water overload. The dry weight was considered equal to the current weight if no ascites was present. In patients with ascites, a review of the patient files was performed to find data on weight after last paracentesis or before recent ascites development. BMI was calculated and weight change that could not be explained by ascites or edema during the previous 6 mo was noted. Dry weight loss was expressed as a percentage of actual body weight. Skinfold thickness at the tricep, bicep, subscapular, and suprailiac sites as well as midarm muscle circumference were measured 3 times by the same research dietitian, and the mean value was used. The sum of the tricep, bicep, subscapular, and suprailiac skinfolds was used to assess percentage body fat according to previously published age- and sex-specific tables (26). This method has been shown to have comparable results with dual energy X-ray absorptiometry in patients with cirrhosis without overt fluid retention (27). Fat-free mass (FFM) was calculated as body weight minus fat mass. Patients were considered malnourished when the triceps skinfold thickness, midarm muscle circumference, or both were below the 5th percentile, according to standard tables for the Swedish population based on age and sex (28), or if BMI (in kg/m²) was < 18.5.

Dietary intake

To assess the subjects’ dietary intake, a 4-d food diary was used as previously described (29). Total daily energy intake is reported in absolute amounts, as a ratio of body weight in kg (energy intake:body weight), and as a ratio of REE (energy intake:REE).

Indirect calorimetry

REE was determined for all subjects in the morning after an overnight fast (10 h) by indirect calorimetry (Deltatrac; Datex, Helsinki, Finland) from 0730 to 0830. To compare REE between the different groups, REE was adjusted for FFM by the use of a linear regression model. Adjusted REE was calculated as the group median REE plus measured REE minus predicted REE, where group median REE is the median absolute REE, measured REE is the metabolic rate measured in each subject, and predicted REE is the calculated rate obtained by using the individual FFM in the linear regression equation generated from the cirrhotic or control group as appropriate (30). Hypermetabolism was defined as a ratio of measured REE to predicted REE > 1.1 (29).

Test meal

On another day, about one week apart from indirect calorimetry, from 0730 to 0800 after an overnight fast, a subgroup of 18 patients with cirrhosis (group A) and all healthy control subjects had a 480 kcal test meal of oatmeal porridge and one cheese sandwich with set amounts of macronutrients (55% of energy as carbohydrate, 31% of energy as fat, and 14% of energy as protein). The test meal is a common kind of breakfast in Scandinavia. The subjects were instructed to eat the meal within 10 min. Blood samples for serum insulin, plasma glucose, and serum C-peptide measurements were drawn from an indwelling cannula at baseline and at 30 min, 60 min, 90 min, 2 h, and 4 h after the meal. In a subgroup of group A—13 patients with cirrhosis (group B)—and all healthy control subjects blood samples were also drawn for plasma leptin and ghrelin analysis at the same intervals.

Blood sample analysis

Blood samples for glucose, insulin, and leptin were drawn after an overnight fast on the day of the test meal from subjects who participated in this part of the study and on the day of indirect calorimetry from all others. Insulin resistance was expressed as homeostasis model assessment index (HOMA-IR) (31). Plasma was immediately separated by centrifugation for 5 min at 1000 × g (4 °C) and then stored at −80 °C until subsequent leptin, ghrelin, or C-peptide analysis. Plasma total ghrelin concentrations were measured by commercial RIA (Linco Research Inc, St Louis, MO) by using 125I-labeled ghrelin as a tracer and ghrelin antiserum specific for total ghrelin. The detection limit for the assay was 93 pg/mL. Ghrelin was expressed in absolute values. Plasma leptin concentrations were measured by using a commercial enzyme-linked immunosorbent assay (Quantikine human leptin, R&D Systems, Oxford, United Kingdom). The
detection limit for the assay was 15.6 pg/mL. Leptin was expressed in absolute values and as a ratio of leptin to weight (leptin:body weight), of leptin to BMI (leptin:BMI), and of leptin to fat in kg (leptin:fat). Patients in subgroup B underwent serological testing for the detection of *Helicobacter pylori* performed according to standard in-house methods.

Statistics

Data are expressed as medians and interquartile ranges (IQRs). The Mann-Whitney *U* test was performed for calculations of differences between groups. For correlation analysis, the Spearman coefficient was calculated. Partial correlation analysis was performed to control for covariates. The chi-square test was used for comparisons between qualitative variables (sex, presence of diabetes, or hypermetabolism). To evaluate plasma glucose, insulin, leptin, and ghrelin changes postprandially, the Friedman’s test was used. When the *P* value was < 0.05, a post hoc analysis with the Wilcoxon’s signed rank test was performed. Multivariate repeated-measures analysis of variance was used to test the interaction between time and group. When the *P* value was < 0.05, the Mann-Whitney *U* test was used to compare the 2 groups at each time point. Stepwise linear regression analysis was used to determine the correlation of independent variables with the energy intake:body weight or the area under the glucose curve (dependent variables), which were transformed into a normal score by using the Blom’s method. All tests were two-tailed and conducted at a 5% significance level. Statistical analysis was done by using SPSS version 11.0.2 (SPSS Inc, Chicago, IL).

RESULTS

The basic characteristics of the patients and healthy control subjects are shown in Table 1. The patients with cirrhosis had higher insulin resistance, leptin, and REE (adjusted for FFM) as well as lower energy intake than did the healthy control subjects (Table 2). No significant differences in any of the variables in Table 2 were observed between the patients with alcoholic and those with nonalcoholic cirrhosis, the patients with Child-Pugh class A and those with Child-Pugh class B or C, the patients with

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Basic characteristics in all subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group A</strong></td>
<td><strong>Group B</strong></td>
</tr>
<tr>
<td><strong>All cirrhotic patients</strong></td>
<td><strong>All patients with cirrhosis in group A</strong></td>
</tr>
<tr>
<td>(n = 31)</td>
<td>(n = 18)</td>
</tr>
<tr>
<td><strong>Age (y)</strong></td>
<td>57 (51–63)</td>
</tr>
<tr>
<td><strong>Sex (M/F)</strong></td>
<td>18/13</td>
</tr>
<tr>
<td><strong>Weight</strong></td>
<td>77 (70–88)</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>26.3 (24.3–29.3)</td>
</tr>
<tr>
<td><strong>Fat (%)</strong></td>
<td>36.2 (31.2–40.3)</td>
</tr>
<tr>
<td><strong>MAMC (cm)</strong></td>
<td>24.7 (21.6–27.7)</td>
</tr>
<tr>
<td><strong>Diabetes (n)</strong></td>
<td>2</td>
</tr>
<tr>
<td><strong>Weight loss (%)</strong></td>
<td>0 (0–1.3)</td>
</tr>
<tr>
<td><strong>Malnutrition (n)</strong></td>
<td>5</td>
</tr>
<tr>
<td><strong>Etiology (n)</strong></td>
<td></td>
</tr>
<tr>
<td>Alcoholic</td>
<td>13</td>
</tr>
<tr>
<td>Viral</td>
<td>5</td>
</tr>
<tr>
<td>PBC</td>
<td>4</td>
</tr>
<tr>
<td>Cryptogenic</td>
<td>6</td>
</tr>
<tr>
<td>Other*</td>
<td>3</td>
</tr>
<tr>
<td>Ascites (n)</td>
<td>6</td>
</tr>
<tr>
<td><strong>MELD score</strong></td>
<td>11 (9–14)</td>
</tr>
<tr>
<td><strong>Child-Pugh score</strong></td>
<td>8 (6–9)</td>
</tr>
<tr>
<td><strong>Encephalopathy grade I (n)</strong></td>
<td>3</td>
</tr>
</tbody>
</table>

* MAMC, midarm muscle circumference; PBC, primary biliary cirrhosis; MELD, Model for End-Stage Liver Disease.

Compared to healthy control subjects. The Mann-Whitney *U* or the chi-square test was used as appropriate for comparisons between groups.

Group A was the subgroup of all cirrhotic patients in which postprandial glucose and insulin were measured.

Group B was the subgroup of group A in which postprandial leptin and ghrelin were measured.

Median; interquartile range in parentheses (all such values).

Fat (expressed as a percentage of body weight or in kg) was calculated from anthropometric data.

Dry weight loss expressed as a percentage of actual body weight during the previous 6 mo (negative values represent weight gain).

Number of malnourished patients (based on a triceps skinfold thickness, midarm muscle circumference 5th percentile, or BMI < 18.5 kg/m²).

Of all patients with cirrhosis, 1 had autoimmune hepatitis, 1 had autoimmune hepatitis and primary sclerosing cholangitis, and 1 had nonalcoholic steatohepatitis (NASH); in group A and B, 1 patient had autoimmune hepatitis and 1 had NASH.
malnutrition and those without malnutrition, and the patients with hepatic encephalopathy and those without hepatic encephalopathy (data not shown).

Fasting leptin was positively correlated with BMI in patients with cirrhosis \( (r = 0.48, P = 0.007) \). Also, leptin was positively correlated with body fat (in kg) in the healthy control subjects \( (r = 0.78, P = 0.008) \) but not in patients with cirrhosis \( (r = 0.18, P = 0.4) \). After control for BMI (partial correlation analysis), fasting leptin was positively correlated with HOMA-IR \( (r = 0.4, P = 0.034) \), negatively correlated with REE \( (r = -0.38, P = 0.042) \), and not significantly correlated with energy intake \( (r = -0.04, P = 0.8) \) in patients with cirrhosis. After control for BMI (partial correlation analysis), fasting leptin was negatively correlated with energy intake \( (r = -0.72, P = 0.029) \) but not to HOMA-IR \( (r = -0.48, P = 0.2) \) or REE \( (r = -0.49, P = 0.2) \) in control subjects.

**Postprandial glucose**

At 30 min postprandially, plasma glucose had risen in both the cirrhosis and the control groups but subsequently remained elevated only in the former (Figure 1). The interaction between time and group for glucose was found to be significant \( (P = 0.037) \). The area under the glucose curve (AUC) and the increase of glucose from baseline to 60 min postprandially were higher in the patients with cirrhosis than in the control subjects [respective median \((IQR): 13.7 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{h}^{-1} (11.9–15)\) compared with \( 10.9 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{h}^{-1} (8.8–11.2)\); \( P < 0.001 \); and respective median \((IQR)\) increases: 54.8% \((22.1–79.6\%\) compared with 20% \((−21.3\%–31.9\%); \( P = 0.002\). The increase of glucose from baseline to 60 min postprandially was negatively correlated with the ratio of energy intake to body weight in the patients with liver cirrhosis \( (r = −0.53, P = 0.023) \) but not in the healthy control subjects \( (r = 0.37, P = 0.3) \). HOMA-IR was positively correlated with the AUC of glucose in the patients with cirrhosis \( (r = 0.75, P < 0.001) \) but not in the control subjects \( (r = 0.16, P = 0.7) \).

**Postprandial insulin**

At 30 min, serum insulin had risen in both the patients with cirrhosis and the control subjects and remained elevated until 2 h postmeal in both groups (Figure 1). The interaction between time and group for insulin was not significant.

**Postprandial C-peptide and serum insulin-to-C-peptide molar ratio**

The interaction between time and group for C-peptide was significant \( (P = 0.035) \). The postprandial C-peptide response was higher in the patients with liver cirrhosis than in the healthy control subjects (Figure 1; AUC of C-peptide: 4.9 \text{ nmol} \cdot \text{L}^{-1} \cdot \text{h}^{-1} \((IQR): 4.2–6.7 \text{ nmol} \cdot \text{L}^{-1} \cdot \text{h}^{-1}\) compared with \( 2.6 \text{ nmol} \cdot \text{L}^{-1} \cdot \text{h}^{-1} \)) at 30 min and 4 h postmeal in both groups (Figure 1). The interaction between time and group for insulin was not significant.

**Postprandial leptin**

The interaction between time and group for leptin was not significant (Figure 2). Similar results were obtained when leptin: BMI or leptin:body weight were used (data not shown).

**Postprandial ghrelin**

Postprandial ghrelin changed significantly compared with baseline only in the healthy control subjects (Figure 2). The interaction between time and group for ghrelin was significant \( (P = 0.015) \). At 4 h, ghrelin was higher in the healthy control subjects than in the patients with liver cirrhosis \( [1176 \text{ pg/mL} \text{ (IQR): 679.3–1692 pg/mL}] \) compared with \( 519 \text{ pg/mL} \) \((379.5–607 \text{ pg/mL}); \( P = 0.021\). The increase of ghrelin from its minimal postmeal value to 4 h postmeal was higher in the healthy control subjects than in the patients with cirrhosis \( [39\% (33.1–48.2\%\) compared with \( 14.2\% (12.8–33.4\%); \( P = 0.005\), and it was
negatively correlated with weight loss in the previous 6 mo in the patients with cirrhosis ($r = -0.66, P = 0.014$). The AUC of ghrelin did not differ significantly between the patients with cirrhosis and the healthy control subjects (data not shown). Postprandial ghrelin concentrations were negatively correlated with glucose and insulin in both the patients with liver cirrhosis and the healthy control subjects (Table 3). The postprandial ghrelin decrease was positively correlated with leptin decrease in the healthy control subjects and negatively in the patients with liver cirrhosis (Table 3). Ghrelin concentrations were not significantly different at any time point between the patients with and those without portal hypertensive gastropathy and between the patients with ($n = 3$) and those without ($n = 10$) serological positivity for *Helicobacter pylori* (data not shown).

**Regression analysis**

Stepwise linear regression analysis was performed for the cirrhosis group with the ratio of energy intake to body weight as the dependent variable. Child-Pugh score, REE, the increase in glucose 60 min postprandially, and the increase in ghrelin from its minimal postmeal value to 4 h postmeal were used as independent variables. Only the increase in glucose 60 min postprandially was found to be independently correlated with energy intake ($\beta = -0.42, P = 0.019$).

In an attempt to identify factors involved in the increased postprandial glucose response, stepwise regression analysis was also performed for the cirrhosis group with AUC of glucose as the dependent variable. Percentage fat mass, HOMA-IR, baseline glucose concentrations, the Child-Pugh score, and the fasting serum insulin-to-C-peptide molar ratio (as a measure of hepatic shunt volume) were used as independent variables. Only insulin resistance expressed as HOMA-IR was found to be independently correlated with the postprandial glucose response ($\beta = 0.82, P = 0.001$) in the patients with cirrhosis.

**DISCUSSION**

In the current study, we observed altered postprandial responses of glucose and ghrelin associated with reduced energy intake and weight loss in patients with liver cirrhosis. The patients with cirrhosis exhibited insulin resistance with higher baseline and postprandial glucose concentrations compared with the healthy control subjects, which agrees with the results of previous studies (3, 7, 12). Although the patients with cirrhosis exhibited both higher fasting insulin and C-peptide concentrations than did the control subjects, indicating increased insulin production in the cirrhotic subjects, the postprandial glucose response was found to be independently related only to insulin
LEPTIN AND GHRELIN IN LIVER CIRRHOSIS

healthy volunteers. We recently reported an increased prevalence of gastrointestinal symptoms (including early satiety) in patients with cirrhosis (35, 36). It is therefore possible that postprandial hyperglycemia results in reduced energy intake by contributing to early satiety and other gastrointestinal symptoms in patients with cirrhosis.

Baseline leptin in patients with cirrhosis was found to be elevated, as previously reported (14–18), and leptin effects on energy intake and REE were disturbed in these patients. Leptin has been shown to increase REE (13), but in a recent study performed in non-cirrhotic individuals, total and free leptin were reported to be negatively and bound leptin positively associated with REE (37). We observed a negative association between total leptin and REE in patients with cirrhosis. It might therefore be hypothesized that the resistance to the effects of leptin in cirrhosis observed in the current study is mediated by a proportional increase in free leptin. However, we did not measure free and bound leptin fractions in our series, which is mandatory to show this. Alternatively, the disturbed associations of leptin with energy intake and REE in cirrhosis might simply indicate disturbed metabolic regulation in these patients, documenting the central role of liver metabolism in whole-body fuel homeostasis. The results of the current study, however, do not support a role of postprandial leptin concentrations in the low energy intake seen in patients with cirrhosis.

Ghrelin concentrations after a meal have not been investigated previously in patients with liver cirrhosis. The patients with cirrhosis had a clearly altered postprandial pattern of ghrelin compared with the control subjects, with an attenuated ghrelin increase at 4 h postmeal. Ghrelin enhances appetite and food intake, and its concentration rises preprandially, thus playing a role in meal initiation (13). Therefore, the low ghrelin observed in the patients with cirrhosis at 4 h postmeal (ie, before expected lunch in our experiment setting) could be involved in the reduced energy intake in these patients. In a recent study, fasting ghrelin was found to be elevated in patients with liver disease compared with healthy control subjects (19). Marchesini et al (5) reported that fasting ghrelin was comparable in patients with cirrhosis and control subjects but increased concentrations were identified in a group of patients with low energy intake and malnutrition. In our study, we were also unable to confirm generally increased fasting ghrelin in patients with cirrhosis. These discrepancies could, at least in part, be explained by different patient selection, control subject selection, or both. Patients in the former study (19) were

resistance. Furthermore, the postprandial increase in glucose was found to contribute independently to the reduced energy intake in the patients with cirrhosis. Decreased hunger and slower gastric emptying were observed in healthy volunteers during induced hyperglycemia (32). Postprandial hyperglycemia has been reported to be associated with increased postprandial upper gastrointestinal symptoms (33, 34) compared with euglycemia in patients with cirrhosis.

![Figure 2](https://example.com/figure2.png)

**FIGURE 2.** Median (half interquartile range) postprandial plasma leptin and ghrelin concentrations in patients with cirrhosis (solid line; n = 13) and in healthy control subjects (dashed line; n = 10). Leptin changed significantly in patients with liver cirrhosis (Friedman’s test, $P = 0.001$) but not in healthy control subjects (Friedman’s test, $P = 0.5$). The interaction between time and group was not significant. Ghrelin changed significantly in the healthy control subjects (Friedman’s test, $P = 0.001$) but not in the patients with liver cirrhosis (Friedman’s test, $P = 0.13$). The interaction between time and group was significant ($P = 0.015$). *Significantly different from concentrations at 4 h, $P = 0.01$ (Wilcoxon’s signed-rank test). §Significantly different from healthy control subjects, $P = 0.021$ (Mann–Whitney U test).

| TABLE 3 |

Spearman correlations of postprandial ghrelin with postprandial glucose, insulin, and leptin variables in patients with liver cirrhosis and healthy control subjects $^1$

<table>
<thead>
<tr>
<th></th>
<th>Ghrelin at 30 min postmeal</th>
<th>Ghrelin decrease at 30 min postmeal</th>
<th>Ghrelin at 90 min postmeal</th>
<th>Ghrelin decrease at 90 min postmeal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients with cirrhosis</td>
<td>Control subjects</td>
<td>Patients with cirrhosis</td>
<td>Control subjects</td>
</tr>
<tr>
<td></td>
<td>$r$</td>
<td>$P$</td>
<td>$r$</td>
<td>$P$</td>
</tr>
<tr>
<td>Glucose increase</td>
<td>−0.63</td>
<td>0.022</td>
<td>0.10</td>
<td>0.748</td>
</tr>
<tr>
<td>at 90 min postmeal</td>
<td>−0.64</td>
<td>0.048</td>
<td>0.04</td>
<td>0.907</td>
</tr>
<tr>
<td>Insulin increase</td>
<td>−0.48</td>
<td>0.112</td>
<td>0.43</td>
<td>0.167</td>
</tr>
<tr>
<td>2 h postmeal</td>
<td>−0.7</td>
<td>0.036</td>
<td>0.67</td>
<td>0.050</td>
</tr>
<tr>
<td>4 h postmeal</td>
<td>0.25</td>
<td>0.443</td>
<td>0.56</td>
<td>0.090</td>
</tr>
<tr>
<td>Leptin decrease</td>
<td>0.14</td>
<td>0.665</td>
<td>−0.54</td>
<td>0.058</td>
</tr>
<tr>
<td>30 min postmeal</td>
<td>0.37</td>
<td>0.209</td>
<td>−0.59</td>
<td>0.035</td>
</tr>
<tr>
<td>90 min postmeal</td>
<td>0.37</td>
<td>0.209</td>
<td>−0.59</td>
<td>0.035</td>
</tr>
</tbody>
</table>

$^1 n = 13$ patients with cirrhosis and 10 control subjects.
transplantation candidates, some had malignancies and were not BMI-matched with control subjects, whereas in the current study, no patients with malignancies were included and BMI-matched control subjects were chosen.

The mechanisms of altered postprandial ghrelin response might involve glucose, insulin, leptin, or all three. Postprandial ghrelin was negatively related to glucose and insulin in both healthy control subjects and patients with cirrhosis, as previously reported (20–22). According to these studies, insulinemia is essential for postprandial ghrelin suppression with glucose having an additional effect (20–22). In our series, the postprandial ghrelin decrease was negatively related to leptin reduction in the patients with cirrhosis. This agrees with earlier data suggesting an inverse relation between leptin and ghrelin and that leptin could be important for suppression of ghrelin (24). Therefore, insulin resistance resulting in high postprandial glucose and insulin might be involved in the low ghrelin observed 4 h postmeal. Thus, it is conceivable that treatment of insulin resistance might reduce the hypoghrelinemia before a meal in patients with cirrhosis, possibly stimulating appetite. Although this is probably not the single most important reason for reduced energy intake in liver cirrhosis, it certainly warrants further studies.

Certain methodologic aspects should be taken into consideration when interpreting the results of the current study. Food intake was assessed by means of food diaries. This is an established method of food intake assessment (29, 37–39), which has been previously utilized in patients with liver cirrhosis (4, 5, 12). However, it is known that both normal-weight and obese subjects may underestimate their dietary intake (39), and it is conceivable that patients with hepatic encephalopathy might also be prone to underreporting when filling in detailed food diaries. In the current study, no patients with encephalopathy grade II or higher were included and food intake was not statistically different between the patients with and those without hepatic encephalopathy grade I. Furthermore, our findings confirm previous studies showing reduced energy intake in patients with cirrhosis (3–5) and reports of a negative correlation between leptin and food intake in healthy subjects (39). Second, in the current study, fasting data were obtained from all subjects but postprandial data were obtained from a smaller subgroup of the main patient population. Although the patients with cirrhosis were carefully matched with the group of healthy control subjects, a type 2 error in the assessment of the postprandial responses cannot be ruled out. Lastly, the current study was a cross-sectional one. Thus, statistical correlations between hormonal disturbances and energy intake or REE in cirrhosis do not necessarily implicate a cause-effect relation.

In conclusion, altered postprandial glucose and ghrelin concentrations correlated with reduced energy intake and weight loss in liver cirrhosis. The effects of leptin on energy expenditure and energy intake seem to be altered in patients with cirrhosis. Insulin resistance might be involved in the altered postprandial glucose and ghrelin responses.

We thank RN Pernilla Jerlstad and dietitian Stine Storsrud for expert technical assistance.

EK contributed to the design of the study, collection and analysis of data, and writing of the manuscript. IB provided advice and consultation on the design of the study and on the writing of the manuscript as well as final review and approval. LO contributed to the analysis of the data and reviewed and approved the final manuscript. EB contributed to the design of the study and writing of the manuscript. None of the authors have a personal or financial conflict of interest.

REFERENCES

26. Dumon JVGA, Womersley J. Body fat assessed from total body density


Effects of probiotic therapy in critically ill patients: a randomized, double-blind, placebo-controlled trial

Cathy Alberda, Leah Gramlich, Jon Meddings, Catherine Field, Linda McCargar, Demetrios Kutsogiannis, Richard Fedorak, and Karen Madsen

ABSTRACT

Background: Multiple organ dysfunction syndrome (MODS) is a major cause of mortality in intensive care units. A breakdown in gut barrier function and immune dysfunction are associated with the onset of MODS. Probiotic bacteria have been shown to modulate intestinal barrier and immune function.

Objective: This study assessed the efficacy of a probiotic compound in a viable and nonviable formulation in modulating intestinal permeability and immune function and preventing the onset of MODS in patients in the intensive care unit.

Design: A double-blind, randomized controlled trial was conducted in the intensive care unit of a tertiary care teaching hospital. Twenty-eight critically ill patients admitted to the intensive care unit were randomly assigned to receive 1 of 3 treatments daily for 7 d: 1) placebo, 2) viable probiotics, or 3) equivalent probiotic sonicates. MODS scores and systemic concentrations of immunoglobulin (Ig) A and IgG were measured on days –1, 4, and 7, and intestinal permeability measurements were taken daily.

Results: The patients responded to viable probiotics with a significantly larger increase in systemic IgA and IgG concentrations than in the patients who received placebo or sonicates (P < 0.05). MODS scores were not significantly affected by probiotic treatment. Over the study period, intestinal permeability decreased in most patients.

Conclusion: Patients receiving viable probiotics show a greater enhancement in immune activity than do patients receiving either placebo or probiotic bacterial sonicates.


KEY WORDS Intestine, multiple organ dysfunction syndrome, Lactobacillus sp., Bifidobacterium, sepsis

INTRODUCTION

Multiple organ dysfunction syndrome (MODS) is a hyperinflammatory state that is a major cause of death in adult intensive care unit (ICU) patients (1–3). The gastrointestinal system appears to play a key role in the pathogenesis of MODS due to a breakdown of intestinal barrier function and increased translocation of bacteria and bacterial components into the systemic circulation (4). This leads to a vicious downward spiral, culminating in immune system dysfunction and multiple organ failure (4).

Intestinal microbes are a major source of systemic infection in postsurgical and trauma patients (5–7). In contrast, endogenous probiotic bacteria of the gut, such as Bifidobacterium and Lactobacillus, play a vital role in maintaining the intestinal mucosal barrier and enhancing immune responses. Feeding probiotics to experimental animals can improve gut barrier function and reduce populations of gram-negative bacteria (8). Because gram-negative organisms account for a significant proportion of infections that arise from the abdominal system, this effect of probiotic bacteria may contribute to their observed benefits (9).

Probiotics have shown efficacy in a wide range of applications, including prophylactic and maintenance treatment of pouchitis (10–14), treatment of radiation-induced diarrhea (15), and adjuvant treatment of ulcerative colitis (16). In mouse models of colitis, live probiotics fed daily enhanced colonic permeability and reduced gut inflammation (8). In vitro studies have shown certain Bifidobacterium strains to release a proteinaceous factor that directly influences epithelial permeability and prevents invasion by potential pathogens (8). Although live probiotics clearly modulate gut immune and barrier function, other studies have shown immunomodulatory effects of probiotic DNA (17). Indeed, some studies have suggested that isolated probiotic bacteria DNA is equally as efficacious in attenuating intestinal inflammation as is treatment with live bacteria (18, 19). The use of isolated bacterial DNA instead of live bacteria in the treatment of sepsis would alleviate the possibility of Lactobacillus sepsis occurring in patients, which, although rare, has been documented (20). Thus, the purpose of this double blind, placebo-controlled, randomized clinical trial was to determine whether the administration of probiotics would maintain gut barrier function and prevent the development of MODS in critically ill, enterally fed patients in the critical care unit, and secondly, to determine whether the effects of bacterial sonicates containing bacterial DNA would be comparable to those effects seen with viable bacteria.

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SUPERTENTS AND METHODS

Study participants

Patients admitted between January and December of 2004 to the ICU of the Royal Alexandra Hospital (Edmonton, Alberta, Canada) were eligible for the study. The following inclusion criteria were applied to patients: 1) >18 y of age, 2) could be fed enterally within 48 h of ICU admission, and 3) anticipated to require enteral nutrition for >48 h. Exclusion criteria included the following: 1) inability to be fed via the gastrointestinal tract; 2) presence of renal failure, pancreatitis, short gut syndrome or pre-existing sacral ulcers; 3) HIV positive; 4) previous bone marrow, lung, or liver transplant; 5) patient had received mannitol, lactulose or anticipated initiation of aforementioned drugs over the upcoming week; or 6) not expected to survive 7 d given their current uncorrectable medical condition. Twenty-eight patients were enrolled in the present study. Consent was provided by the patient or, if the patient was unable to, by immediate family members.

The Ethics Committee for Medical Research at the University of Alberta approved the study protocol. Before their admittance into the study, written and oral informed consent were provided from all study participants or their relatives. The study was performed in accordance with International Conference on Harmonisation Good Clinical Practice guidelines based on the Declaration of Helsinki.

Treatment

Patients who met the inclusion criteria and provided consent were randomly assigned to one of three treatment groups: 1) placebo; 2) viable probiotics—2 sachets daily; and 3) bacterial sonicates (not viable). Each sachet of probiotics (VSL#3; VSL Pharmaceuticals, Ft Lauderdale, FL) contained 900 billion viable lyophilized bacteria consisting of 4 strains of Lactobacillus (L. casei, L. planatarum, L. acidophilus, and L. delbrueckii subsp. Bulgaricus), 3 strains of Bifidobacterium (B. longum, B. breve, and B. infantis) and Streptococcus salivarius subsp. Thermophilus. Bacterial sonicates were prepared by rehydrating probiotic sachets in sterile water and sonicating twice for 30 s. Homogenates were centrifuged at 100,000 g for 10 min at 4°C and filtered through a 0.22 μm filter to remove all live bacteria. Bacterial DNA was present in the sonicate. A sample was taken for culture to ensure no live bacteria remained in the supernatant, and the supernatant immediately frozen at −70 °C until use. All treatment groups also received a polymeric enteral formula containing 22 g fiber/1000 mL, which includes 10 g fructooligosaccharides/1000 mL and 12 g of a patented soluble and insoluble fiber blend (Jevity Plus; Abbott Laboratories, Columbus, OH). The treatment solutions were identical in appearance.

Study design

This was a single center double-blind, placebo controlled trial. Enteral nutrition was provided to study patients within 48 h from the time of ICU admission. Enteral feeds were initiated and progressed according to Capital Health Region ICU protocol. By protocol, nasoenteric feeds were started at 25 mL/h and increased by 25 mL/h every 4 h until the target rate was achieved. When gastric residual volumes exceeded 150 mL, prokinetic agents were initiated and feeds were resumed and advanced until the target rate was achieved. All study treatments were administered twice daily at 0900 and 2100. Within 60 min of reconstitution, the study treatment and placebo preparations were dispensed in identical packaging and administered to the patient via a feeding tube. The probiotics group received 2 sachets of probiotics twice daily providing a total of 9 x 10^11 bacteria. Bacterial sonicate treatments were prepared from an equivalent dosage; on preparation, they were immediately frozen, transported, and infused to the patients within 1 h of thawing. Patients received the study treatment for 7 consecutive days. If the patient was to discontinue enteral nutrition or was ready to be transferred out of the ICU before the study completion, the study was discontinued prematurely. All patients in the study received concomitant therapy, including antibiotics, as considered appropriate by the attending physician.

Apache II scores

Acute Physiology and Chronic Health Evaluation II (APACHE II) scores were calculated utilizing data obtained during the 24 h before initiation of enteral nutrition (21).

Nutritional assessment

Energy requirements were calculated as 25–30 kcal/kg and protein requirements as 1.2–1.5 g/kg protein. Daily energy and protein intake were recorded. Body mass index was calculated by the formula weight (in kg)/height^2 (in m) (2), and subjective global assessment was assessed at the initiation of enteral nutrition.

Indirect calorimetry

On achieving the target rate of enteral feeding, an indirect calorimetric measurement was performed to confirm the adequacy of enteral nutrition. Patients were assessed by using a Sensormedics Deltrac II indirect calorimeter (Sensormedics, Yorba Linda, CA) for ≥20 min. The patients did not receive any analgesia, stimulation, or undergo any ventilatory changes for 30 min either before the test or during the measurement. Acceptable variations in VO_2 (volume of oxygen utilized, in mL/min) and VCO_2 (volume of carbon dioxide produced, in mL/min) were defined as <15%, and acceptable variation in respiratory quotient was defined as <10%. Measurements that exceeded these limits were not interpreted. Energy requirements were reassessed based on the indirect calorimetric results, and enteral feeding rates were adjusted to meet resting energy expenditures.

Outcome measures

Multiple Organ Dysfunction Score

A MODS score was calculated on day −1, 4, and 7 of the study. Briefly, the parameters used to calculate MODS for each individual system were as follows: 1) respiratory (partial pressure of oxygen/fraction of inspired oxygen), 2) renal (serum creatinine), 3) hepatic (bilirubin), 4) cardiovascular (pressure adjusted heart rate), 5) hematologic (platelets), and 6) neurologic (Glasgow Coma Scale) (22).

Biochemical analysis

C-Reactive protein (CRP), immunoglobulin (Ig) A, and IgG baseline measurements were made on day −1, before initiation of
the study treatment. Measurements were repeated at the completion of the study (day 7) after a 6 h urine collection for intestinal permeability. In those cases where a subject completed the study before Day 7, blood was drawn at the conclusion of the last intestinal permeability collection.

Diarrheal episodes

Diarrheal episodes were measured daily by the Hart & Dobb diarrhea scale. Diarrhea was defined as a score of ≥12 in a 24 h period (23). Diarrhea incidence was calculated by the number of days with Hart and Dobb Score of ≥12 divided by the number of days patients received treatment and enteral nutrition.

Intestinal permeability

Intestinal permeability was measured daily for 7 d by using a standardized protocol (24). The first measurement was performed on Day −1 before the patient received treatment. A syringe containing 7.5 mL lactulose was sent daily to the bedside with 2 g mannitol. The mannitol was reconstituted with 20 mL distilled water and administered enterally daily during the ICU stay. Twenty mL water was given to rinse the feeding tube after administration of the sugar solution. Feeding with enteral preparations was temporarily interrupted during administration of the sugar solution but was immediately resumed after the rinse solution. The excreted portion of each sugar marker was collected for 6 h in urine via a standard urinary catheter collecting system to which gentamicin was added. Collected urine was placed in a bottle containing 5 mL 10% thymol. The collected urine was drained from the catheter bag every quarter hour. The collection jug was kept on ice at the bedside for the 6 h collection time. The collection jug was refrigerated at 4 °C and then stirred, before taking two 15-mL aliquots of urine. All samples were frozen to −70 °C within 24 h. Measurement of the urinary concentration of sugars was made by using standardized HPLC methodology (24).

Statistical analysis

The data were analyzed by using the statistical software program SPSS 12.0, Statistical Package for the Social Sciences (SPSS Inc, Chicago, IL). Independent t tests were performed on all baseline data between groups. Differences in variables at baseline and after treatment were assessed with a repeated-measures analysis of variance that included a time × treatment interaction. Tukey’s post hoc tests were used to assess differences between the treatment groups. Differences between means were evaluated by using analysis of variance or paired t tests where appropriate. Data were further analyzed with a Bonferroni adjusted t test for multiple comparisons. The Mann-Whitney U test was used to compare nonparametric data. Intestinal permeability measures were reported as the lactulose mannitol ratio (LMR). LMR results were converted to their natural log (ln) values to normalize the distribution for analysis. Cohorts were compared for daily changes in permeability through the use of linear mixed-effects model, a technique that allows for comparisons between the means of cohorts, unit changes in permeability per unit change per day, and inclusion of effects of daily changes of physiologic dysfunction and accounts for individual variability between patients. Reported P values were two-tailed; P values < 0.05 were considered significant for all statistical tests. A total sample size of 45 subjects was calculated based on an α of 0.05 and power of 90% by using independent t test calculations for differences in intestinal permeability. Interim analysis was required as numerous subjects stopped enteral nutrition before study completion.

RESULTS

Participant characteristics

Twenty-eight patients were enrolled into the trial (n = 9 for placebo, 10 for viable probiotics, and 9 for probiotic sonicates). No significant differences in age, sex, APACHE II scores, MODS, body mass index, or use of antibiotics were observed between the groups. (Table 1)

Nutritional variables

Patients in the treatment groups were not significantly different in terms of nutritional status (Table 2). Mean daily energy intake was compared with energy requirements derived from indirect calorimetric measurements as described in the methodology section. Mean protein intakes were compared with protein requirements calculated by formulaic methods. No significant differences existed between treatment groups for mean energy and protein intake. The 2 most common reasons for interrupting enteral nutrition included temporary cessation for medical procedures or increased gastric residuals >150 mL as per ICU enteral feeding protocol.

Development of Multiple Organ Dysfunction Syndrome

MODS analysis was completed on days −1, 4, and 7. No statistically significant differences in MODS scores were observed either within groups from days −1 to 7 or between the groups on days −1, 4, or 7. In the group receiving viable probiotics, 50% (5 of 10) of the patients transferred out of the ICU on day 4 of the study (Table 3). In the placebo group, 33% of patients (3 of 9) transferred out of the ICU on day 4 of the study, and in the group receiving probiotic sonicates, 22% (2 of 9) transferred out of the ICU on day 4. No significant difference in the proportion discharged from the ICU was observed between the groups.

Immune variables

The patients who received viable probiotics showed significantly greater increases in IgG and IgA than did the patients who received placebo or probiotic sonicate (P < 0.05) (Figure 1). The increase in IgG and IgA concentrations was not significantly different in the patients who received sonicates compared with the patients who received placebo (P = 0.64). Overall, there was a significant increase in IgG and IgA concentrations over the treatment period for all patients (P < 0.05). A significant decline in CRP concentrations occurred in all treatment groups (P < 0.05). No significant differences in the change in CRP values were observed between the placebo (73 ± 28), probiotic (9 ± 35), and probiotic sonicates (63 ± 25) groups.

Incidence of diarrhea

Diarrhea incidence was calculated by number of days with Hart and Dobb Score of ≥12 divided by the number of days patients received treatment and enteral nutrition. Patients who received placebo had a 23% incidence of diarrhea compared with
14% in patients who received viable probiotics and 12% in patients who received probiotic sonicates.

**Intestinal permeability**

On entry into the study, most patients had an elevated lactulose-to-mannitol ratio, indicative of increased small intestinal permeability (placebo: 6 of 7, 86%; viable probiotics: 6 of 7, 86%; and probiotic sonicates: 3 of 8, 38%). Individual patient small intestinal permeability measurements before initiation of therapy and at the end of the treatment period are shown in Figure 2. The average daily intestinal permeability measurements are shown in Figure 3. Overall, most patients showed a significant decrease in small intestinal permeability over the treatment period ($P < 0.003$; Figure 2). No significant difference in intestinal permeability in response to treatment was observed between the patients receiving live probiotics, probiotic sonicates, and placebo ($P = 0.06$). A positive correlation was observed between energy intake and intestinal permeability ($P < 0.01$); neither age nor APACHE II scores correlated with intestinal permeability results.

**Adverse events**

No adverse effects of placebo or probiotic therapy were noted at any time during the study. One patient was switched to total parenteral nutrition during the study because of bowel obstruction. At the conclusion of the study, it was determined that the patient had received viable probiotic therapy. No patients in the treatment group developed *Lactobacillus*-induced sepsis.

**TABLE 1**

Demographics and clinical variables of study participants by treatment group at study entry

<table>
<thead>
<tr>
<th></th>
<th>Placebo group ($n = 9$)</th>
<th>Viable probiotics group ($n = 10$)</th>
<th>Probiotic sonicates group ($n = 9$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (y)</strong></td>
<td>64.9 ± 16.9$^2$</td>
<td>60.4 ± 17.9</td>
<td>66.6 ± 18.9</td>
</tr>
<tr>
<td><strong>Sex (M/F)</strong></td>
<td>4/5</td>
<td>5/5</td>
<td>3/6</td>
</tr>
<tr>
<td><strong>Reason for ICU admission</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medicine [n (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory</td>
<td>6 (66.7)</td>
<td>5 (50)</td>
<td>4 (44.4)</td>
</tr>
<tr>
<td>Cardiac</td>
<td>0</td>
<td>1 (10)</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td>Neurological</td>
<td>1 (11.1)</td>
<td>1 (10)</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td>Sepsis</td>
<td>1 (11.1)</td>
<td>1 (10)</td>
<td>0</td>
</tr>
<tr>
<td>Overdose</td>
<td>0</td>
<td>0 (10)</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td>Surgical [n (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thoracics (postop)</td>
<td>0</td>
<td>1 (10)</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td>Trauma</td>
<td>1 (11.1)</td>
<td>1 (10)</td>
<td>0</td>
</tr>
<tr>
<td><strong>MODS score$^3$</strong></td>
<td>3.8 ± 1.6</td>
<td>4.6 ± 3.9</td>
<td>4.0 ± 1.8</td>
</tr>
<tr>
<td><strong>APACHE II</strong></td>
<td>15.9 ± 4.2</td>
<td>19.1 ± 4.1</td>
<td>17.3 ± 4.4</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>25.8 ± 5.2</td>
<td>23.5 ± 5.8</td>
<td>28.8 ± 7.6</td>
</tr>
<tr>
<td><strong>Types of antibiotics (no./d)</strong></td>
<td>1.4 ± 1.0</td>
<td>1.5 ± 0.9</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td><strong>Survival in ICU (n)</strong></td>
<td>8</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

$^1$ MODS, multiple organ dysfunction syndrome; ICU, intensive care unit; APACHE II, acute physiology and chronic health evaluation II. Independent *t* tests were performed on all baseline data between the groups, and variables were assessed with ANOVA. No significant differences were observed between the groups.

$^2$ $\bar{x} \pm$ SD (all such values).

$^3$ Variables used to calculate MODS included partial pressure of oxygen/fraction of inspired oxygen, serum creatinine and bilirubin, pressure-adjusted heart rate, platelet count, and the Glasgow Coma Scale.

$^4$ Determined by energy intake from enteral nutrition/energy requirements assessed through indirect calorimetry.

$^5$ Determined by grams protein consumed via enteral nutrition/grams protein required from formulaic assessment of 1.2–1.5 g protein · kg$^{-1}$ · d$^{-1}$ (mean).

**TABLE 2**

Nutritional variables of the study participants by treatment group

<table>
<thead>
<tr>
<th></th>
<th>Placebo group ($n = 9$)</th>
<th>Viable probiotics group ($n = 10$)</th>
<th>Probiotic sonicates group ($n = 9$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGA ($n$) $^2$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>6</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Energy intake (kcal/d)</strong></td>
<td>1406 ± 261$^4$</td>
<td>1199 ± 509</td>
<td>1388 ± 417</td>
</tr>
<tr>
<td><strong>Energy requirements met (%)$^5$</strong></td>
<td>87.3 ± 10.4</td>
<td>74.6 ± 13.3</td>
<td>82.6 ± 22.8</td>
</tr>
<tr>
<td><strong>Protein intake (g/d)</strong></td>
<td>65.7 ± 12.3</td>
<td>56.0 ± 23.6</td>
<td>64.8 ± 19.1</td>
</tr>
<tr>
<td><strong>Protein requirements met (%)$^5$</strong></td>
<td>87.3 ± 10.4</td>
<td>64.5 ± 18.8</td>
<td>74.3 ± 19.6</td>
</tr>
</tbody>
</table>

$^1$ Independent *t* tests were performed on all baseline data between the groups, and variables were assessed with ANOVA. No significant differences were observed between the groups.

$^2$ SGA, subjective global assessment. A, well-nourished; B, moderately malnourished; and C, severely malnourished.

$^3$ $\bar{x} \pm$ SD (all such values).

$^4$ Determined by energy intake from enteral nutrition/energy requirements assessed through indirect calorimetry.

$^5$ Determined by grams protein consumed via enteral nutrition/grams protein required from formulaic assessment of 1.2–1.5 g protein · kg$^{-1}$ · d$^{-1}$ (mean).
A patient in each of the 3 treatment groups died during their ICU admission. The causes of death for the patients who died in ICU included respiratory failure (one patient on sonicates), congestive heart failure (one patient on viable probiotics), and myocardial infarction (one patient on placebo). The deaths in the treatment groups occurred after the probiotic therapy had been discontinued. The patient randomly assigned to viable probiotics died on day 9 of ICU admission, and the patient randomly assigned to sonicates died on day 128 of ICU admission.

**DISCUSSION**

The present pilot study used a double-blind, placebo-controlled, randomized design to determine the effects of viable probiotics and probiotic sonicates on the development of MODS in critically ill, enterally fed patients. Overall, the patients who received viable probiotics showed a greater enhancement in immune activity and reduction in intestinal permeability than did the patients who received either placebo or sonicates.

MODS scores calculated over the first 24 h of ICU admission correlate strongly with ICU mortality rates (25). In validation studies, ICU mortality rates have been shown to be 100% for MODS scores $\leq 20$, 50% for those patients with scores between 13 and 16, and $\leq 5\%$ for those patients with scores between 1 and 4. Patients entered into the current study had pretreatment MODS scores ranging from 2 to 14. Logistic regression analysis has shown that an increase in MODS scores of one point increases the odds of death by 1.59. Patients who received viable probiotics had an average reduction of 1.2 in their MODS scores after 3 days of treatment.

Probiotics modulate the innate and adaptive immune system in a dose- and strain-dependent manner (26, 27). In particular, some *Lactobacilli* and *Bifidobacteria* strains have been shown to induce the production of secretory IgA and IgG (26, 28, 29). High concentrations of IgA activity in the gut are crucial to maintaining a barrier against pathogenic bacterial translocation, especially of gram-negative organisms (30). In our study, administration of viable probiotics significantly increased IgA and IgG production. A similar finding in a mouse model was reported by Galdeano and Perdigon (27), who showed that viable *L. casei* and *L. acidophilus* increased the number of IgA cells in the intestine of mice to a much greater extent than did nonviable bacterial cells. However, in contrast to our results, 2 studies showed no increases in IgA in surgical patients who consumed ProViva, an oatmeal-based drink containing *Lactobacillus plantarum* 299v or a probiotic compound containing *Lactobacillus acidophilus* TABLE 3

<table>
<thead>
<tr>
<th>Day</th>
<th>Placebo group</th>
<th>Viable probiotics group</th>
<th>Probiotic sonicates group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MODS$^2$</td>
<td>$n$</td>
<td>MODS$^2$</td>
</tr>
<tr>
<td>−1</td>
<td>3.8 ± 1.6$^3$</td>
<td>9</td>
<td>4.6 ± 3.8</td>
</tr>
<tr>
<td>4</td>
<td>4.1 ± 1.5</td>
<td>9</td>
<td>3.4 ± 2.7</td>
</tr>
<tr>
<td>7</td>
<td>4.2 ± 1.6</td>
<td>6</td>
<td>4.0 ± 1.9</td>
</tr>
</tbody>
</table>

Data were analyzed by using a linear mixed-effects model for periods between days −1 and 4 and between days 4 and 7. No significant differences or time × treatment interactions were observed.

Variables used to calculate MODS included partial pressure of oxygen/fraction of inspired oxygen, serum creatinine and bilirubin, pressure-adjusted heart rate, platelet count, and the Glasgow Coma Scale.

$^3$ x ± SD (all such values).

FIGURE 1. Mean (±SD) serum immunoglobulin (Ig) G and IgA concentrations in the 3 groups of patients at day −1 (■) and day 7 (■) of treatment. The patients who received viable probiotics ($n = 10$) showed significantly greater increases in IgG and IgA than did the patients who received placebo ($n = 9$; $P < 0.04$) or probiotic sonicates ($n = 9$; $P < 0.05$). No significant difference in the change in IgG and IgA concentrations was observed between the patients who received sonicates and the patients who received placebo. A significant increase ($P < 0.05$) in IgG and IgA concentrations was observed over the treatment period for all patients. Change (difference between day 7 and day −1) was significantly different from that of the placebo and sonicate groups, $P < 0.05$ (ANOVA with Bonferroni-adjusted $t$ test for multiple comparisons).
Intestinal permeability was assessed daily by measuring the excretion of mannitol and lactulose in urine by HPLC. The first measurement was performed on day −1 before the patients received treatment. Results are reported as a ratio between excreted lactulose and mannitol (lactulose-to-mannitol ratio, LMR) in individual patients who received placebo (A: n = 7), viable probiotics (B: n = 7), and probiotic sonicates (C: n = 8). Independent t tests were performed on all baseline data between the groups. Differences in permeability at baseline and after treatment were assessed with a repeated-measures ANOVA that included a time × treatment interaction. Tukey’s post hoc tests were used to assess differences between the treatment groups. LMRs were converted to their natural log values to normalize the distribution for analysis. Cohorts were compared for daily changes in permeability by a linear mixed-effects model that compared means of cohorts, unit changes in permeability per unit change per day, inclusion of effects of daily changes of physiologic dysfunction, and accounted for individual variability between patients. A significant decrease in small intestinal permeability over time was observed in all treatment groups (P < 0.003).

Intestinal permeability was assessed daily by measuring excretion of mannitol and lactulose in urine by HPLC. The first measurement was performed on day −1 before the patient received treatment. Results are reported as a ratio between excreted lactulose and mannitol (lactulose-to-mannitol ratio, LMR) in the groups. Independent t tests were performed on all baseline data between groups. Differences in permeability at baseline and after treatment was initiated were assessed with a repeated-measures ANOVA that included a time × treatment interaction. Tukey’s post hoc tests were used to assess differences between the treatment groups. LMRs were converted to their natural log values to normalize the distribution for analysis. No significant difference in intestinal permeability was observed between the groups on day −1 (before receiving treatment).
La5, Lactobacillus bulgaricus, Bifidobacterium lactis Bb-12, and Streptococcus thermophilus (31). Whether these negative results are due to the selected strain or dose of bacteria, the duration of treatment, the type of patients studied (undergoing major surgery), or the possibility of variability in consumption of the study compound before surgery by patients (and corresponding lack of control over possible consumption of over-the-counter probiotic-containing compounds by patients taking placebo) remains to be determined. However, several factors are known to be important in probiotic therapy, with strain selection, time point when therapy is initiated, and dose being critical (32).

CRP, commonly used as a marker of systemic inflammation (33), is an acute-phase protein produced by the liver and by endothelial cells (34). CRP inhibits the production of proinflammatory cytokines and chemokines, including tumor necrosis factor α and interferon γ (35) and also demonstrates significant antimicrobial activity (36). Although most of the patients in the present study showed a reduction in CRP concentrations over the treatment period, those patients who received viable probiotics had a lesser decline in CRP concentrations than did those patients who received either placebo or bacterial sonicates. Interestingly, a recent study by Viljanen et al. (37) showed that Lactobacillus GG treatment of infants with atopic eczema-dermatitis and cow milk allergy resulted in higher concentrations of CRP than did placebo treatment. CRP has been shown to have a protective effect in preventing the onset of disease in lupus-prone transgenic mice (38), which suggests that the role of CRP in sepsis is likely more complex than simply being a nonspecific marker of inflammation. Further, a probiotic-induced maintenance of CRP concentrations may actually be beneficial in treating systemic infection through its antimicrobial actions (36). Additional studies are necessary to clearly define the role of CRP in sepsis and also the effect of probiotic bacteria in modulating CRP production.

A decrease in intestinal permeability was observed over time in most patients in all 3 groups. Probiotics have been shown to have positive effects in reducing small intestinal permeability (39, 40) but also to be ineffective (41). It is interesting that colonic permeability has not routinely been assessed in critically ill patients, because it is possible that the primary effects of probiotic therapy may be seen in the maintenance of colonic, rather than small intestinal, permeability. However, a recent systematic review suggests that the addition of probiotics to enteral nutrition can enhance the beneficial effects of enteral nutrition on patient outcomes, including the modulation of inflammation and systemic immunity, as was seen in the present study (39). It is clear that the therapeutic potential of probiotics to prevent increases in intestinal permeability and its complications requires further investigation.

Administration of both viable probiotics and probiotic sonicates decreased the incidence of diarrhea. However, absolute power was low at 0.176. The effect size of 0.065 may also account for the statistical insignificance of the finding. Previously, 64 patients per treatment group were randomly assigned to receive placebo or Saccharomyces boulardii with their tube feedings (40). The investigators concluded that S. boulardii decreased the incidence of diarrhea by 4.7% in critically ill tube-fed patients (40).

The percentage of energy requirements achieved with enteral nutrition was not significantly different between the groups. Difficulties establishing enteral nutrition are greater in patients with higher severities of illness because of gut dysmotility and hypoperfusion to the gastrointestinal tract. In the current study, the most frequent reasons for stopping or interrupting enteral feeds were for medical procedures and for high gastric residuals. Two of the 28 patients received <50% of the prescribed energy and protein provisions. In light of the findings on intestinal permeability and immune variables, the most advantageous route seems to be enteral nutrition administered with probiotic therapy. However, the absolute power and effect size were low in all outcome measures, indicating that the study needs to be replicated in a larger sample size to be conclusive. The second limitation of the study was the heterogeneity of the ICU patients enrolled, because the study subjects included medical, surgical, and trauma patients. Future studies may consider attempting to enroll patients with a higher severity of illness but with a similar disease profile. In addition, larger studies are required to confirm the efficacy and safety of probiotics in preventing the onset of MODS in ICU patients.

REFERENCES


Arachidonic acid– and docosahexaenoic acid–enriched formulas modulate antigen-specific T cell responses to influenza virus in neonatal piglets1–3

Josep Bassaganya-Riera, Amir J Guri, Alexis M Noble, Kathryn A Reynolds, Jennifer King, Cynthia M Wood, Michael Ashby, Deshanie Rai, and Raquel Hontecillas

ABSTRACT

Background: Whereas the immunomodulatory effects of feeding either arachidonic acid (AA) or docosahexaenoic acid (DHA) separately have been previously investigated, little is known about the immunomodulatory efficacy of AA or DHA when they are fed in combination as infant formula ingredients.

Objective: The objective of this study was to investigate the ability of AA- and DHA(AA/DHA)-enriched infant formula to modulate immune responses in the neonate in response to an inactivated influenza virus vaccine.

Design: Neonatal piglets (n = 48) were weaned on day 2 of age and distributed into 16 blocks of 3 littermate piglets each. Within each block, piglets were randomly assigned to a control formula, AA/DHA-enriched formula (0.63% AA and 0.34% DHA), or sow milk for 30 d. On day 9, 8 blocks of piglets were immunized with an inactivated influenza virus vaccine. On days 0, 9, 16, 23, and 30 after weaning, we measured influenza virus–specific T cell proliferation and phenotype of T subsets in peripheral blood. A delayed-type hypersensitivity reaction test was administered on day 28. Cytokine messenger RNA expression was determined by quantitative real time reverse transcriptase–polymerase chain reaction on day 30.

Results: The influenza virus–specific CD4+ and CD8+ T cell ex vivo lymphoproliferative responses were significantly lower on day 23 after immunization in piglets receiving dietary AA/DHA supplementation and sow milk than in those receiving the unsupplemented control formula. The immunomodulatory effects of AA/DHA-enriched formulas were consistent with up-regulation of interleukin 10 in peripheral blood mononuclear cells.


KEY WORDS Arachidonic acid, docosahexaenoic acid, immunity, growth, piglets

INTRODUCTION

The nutritional requirements of essential fatty acids (ie, linoleic and α-linolenic acids) for growth and development are well established. Breast milk is the best source of nutrition for young infants; however, when it is not available, infant formulas are the only source of nutrition for many infants during the first 4–6 mo of life. Therefore, the minimum concentrations of 29 nutrients, including linoleic acid, are tightly regulated in the United States under the provisions of the Infant Formula Act of 1980, its 1986 amendments, and the Code of Federal Regulations (21 CFR). In addition, new ingredients added to infant formulas are regulated under the Food, Drug and Cosmetic Act of 1938. Matching the ingredient composition of infant formulas with the nutritional composition of human milk or breastfeeding performance is a widely used strategy for improving infant formulas.

Human breast milk contains, in addition to essential fatty acids, other polyunsaturated fatty acids (PUFAs) such as arachidonic acid (AA), docosahexaenoic acid (DHA), and conjugated linoleic acid (CLA). The presence of these PUFAs in human milk suggests that they may be required for optimal health of infants and children. Whereas AA and DHA can be synthesized by term and preterm infants born at 33 wk gestation (1), the limited rates of desaturation and elongation from linoleic and linolenic acids to AA and DHA, respectively, in young infants may not meet the needs for optimal infant growth and development (2–5). Hence, whether long-chain PUFAs are essential or conditionally essential nutrients required for optimal infant nutrition remains controversial (1, 6).

Supplementation of infant formulas with AA and DHA is believed to favorably modulate the development of the nervous system (7), the retina (8), the auditory system (9), and the digestive system (10). Some preliminary data also suggest a benefit of AA and DHA on immune system development and function in the early neonatal period. For instance, Field et al (11) showed that, in preterm infants receiving AA and DHA supplementation, the lymphocyte populations, the phospholipid composition of lymphocytes, and markers of immune cell maturity were similar to those in breastfed infants. Moreover, the concentrations of

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2 Supported by a grant from Mead Johnson Nutritionals/Bristol Myers Squibb (to JBR) and by the Nutritional Immunology & Molecular Nutrition Laboratory.
3 Reprints not available. Address correspondence to J Bassaganya-Riera, Nutritional Immunology & Molecular Nutrition Laboratory, 319 Wallace Hall, Virginia Tech University, Blacksburg, VA 24060. E-mail: jbassaga@vt.edu. Received July 20, 2006. Accepted for publication October 26, 2006.

interleukin 10 (IL-10) in infants fed AA- and DHA- (AA/DHA) enriched formula resembled those in breastfed infants, whereas IL-10 production was suppressed in infants fed the control formula (11). Even though the effects of dietary AA and DHA supplementation on immune function were investigated previously in adults and found to elicit opposing immunomodulatory actions (12, 13), little is known about their immunomodulatory efficacy when fed in combination in the early neonatal period. The primary goal of the current study was to determine whether AA/DHA-enriched formulas would modulate adaptive T cell responses in the neonate. To achieve this goal, we used a neonatal piglet model, because piglets are comparable in size, physiology, and immunity to human infants.

**MATERIALS AND METHODS**

**Experimental design**

Ten pregnant (primiparous or first parity) Yorkshire × Landrace sows were purchased from a herd with low or null influenza virus antibody titers and transported to the infant formula testing facility at Virginia Tech University. The original breeding herd was also free of other pig pathogens, such as porcine respiratory and reproductive virus, *Mycoplasma hyopneumoniae*, pseudorabies virus, *Salmonella typhimurium*, or porcine parvovirus. The sows were housed in a temperature- and humidity-controlled facility. Airflow was adjusted on the basis of the sows’ comfort level and the age of the piglets. Before parturition, sows were fed 2.00–2.20 kg of sow ration each day at 0900. After parturition, sows were twice daily (ie, 0900 and 1700) fed increasing amounts of sow ration starting at 2.5 kg/feeding on day 1 and ending at 3.84–4.20 kg/feeding on day 30.

The temperature in the infant formula testing facility was set between 70 and 72 °F throughout the course of the study, and local heat was provided by heat lamps. In the adjustable lactation crates, zone heating was provided by heat lamps, which were turned on while sows were farrowing. In addition, the umbilical cords were tied off and disinfected with iodine immediately after birth. Between 6 and 24 h after parturition, neonatal piglets were given 0.5 cc penicillin intramuscularly, ear tagged, and weighed. Naxcel (0.5 cc at 5 mg/kg) was administered intramuscularly every other day starting on day 1. On day 2, 48 neonatal piglets with an initial body weight of 1.5–1.8 kg were selected from a total of 92 neonatal piglets born in the infant formula testing facility. All the piglets were given 1 cc iron dextran (100 mg; intramuscularly) to prevent anemia, and, in line with the policy of reducing the number of animals used for research, the piglets as well as the sows not included in this study were returned to a facility. All of the animals were handled according to the practices of animal care established by the Virginia Tech and Bristol-Myers Squibb animal care and use committees. The animal care and use protocols met or exceeded the guidelines of the National Institutes of Health Office of Laboratory Animal Welfare and the policy of the Public Health Service.

**Dietary treatments**

The dry formula was prepared by mixing 150 g of a concentrated, fat-free basal mix with 50 g fat blend by using a dry formula mixer. For the AA/DHA-enriched formula, the AA and DHA were mixed with the fat blend under a nitrogen blanket before the fat blend was mixed with the rest of the ingredients. The dry infant formula (200 g; Table 1) was reconstituted with 1 L deionized sterile water in a heavy-duty homogenizer for 2 min. The reconstituted formula provided 4074 kJ or 970 kcal/kg. It is estimated that human breast milk provides a mean value of 670 kcal/kg from 1–24 mo of lactation (15, 16). This formula met or exceeded the requirements for growing piglets as set by the National Research Council (17), and it can support the growth of piglets from day 1 to day 30 of age. The piglet formula was prepared 3–5 times/d and stored at 4 °C, and the semiautomatic feeders were refilled every 4 h. The ratio of AA to DHA in the enriched formula was maintained at 1.9:1. AA was provided in the form of a triacylglycerol oil source rich in AA (Martek Biosciences Corp, Columbia, MD) generated by the fungus *Mortierella alpina*, which contains AA at 40% of fatty acids. Other fatty acids found in the source of AA include palmitic acid (5–15%), stearic acid (10–20%), oleic acid (12–39%), linoleic acid (5–15%), and linolenic acid (2.5–5%). DHA was provided in the form of a triacylglycerol oil source rich in DHA that is produced commercially (Martek Biosciences) from *Cryptothecodium colahi*, a marine dinoflagellate, which contains DHA as 40% by weight of fatty acids, with the balance being myristic acid (10–20%), palmitic acid (15–20%), oleic acid (10–30%), and linoleic acid (1–5%). In addition to the fungal or algal oils, both products contain high-oleic sunflower oil, tocopherols, and ascorbyl palmitate as ingredients. Both sources of AA and DHA used in this study are Generally Recognized as Safe (GRAS) by the Food and Drug Administration (GRAS Notice no. GRN 000041). GRAS status means that a food ingredient is not anticipated to cause harm at the approved concentrations.

**Immunization with an inactivated influenza virus vaccine**

On day 9, 8 blocks of 3 pigs each were immunized with the use of an inactivated influenza virus vaccine in order to assess the kinetics of antigen-specific immune responses on days 0, 9, 16, 23, and 30 after weaning and the delayed-type hypersensitivity reactions to influenza virus antigens on day 28. Influenza viruses (VR-1469 strain), propagated in Madin-Darby Canine Kidney (MDCK) cells as previously described (18), were obtained from...
TABLE 1
Composition of dry formulas before reconstitution with water

<table>
<thead>
<tr>
<th>Ingredient (g/kg diet)</th>
<th>Control formula</th>
<th>AA/DHA–enriched formula</th>
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<tr>
<td>Non-fat milk powder2</td>
<td>518.16</td>
<td>518.16</td>
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<tr>
<td>Lactalbumin2</td>
<td>125</td>
<td>125</td>
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<tr>
<td>Lactose</td>
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<td>64.407</td>
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<tr>
<td>Citric acid</td>
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<td>10</td>
</tr>
<tr>
<td>Vitamin mix (AIN-93)³</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Ferric citrate</td>
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<td>0.58</td>
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<tr>
<td>Zinc acetate</td>
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<td>0.25</td>
</tr>
<tr>
<td>Cupric carbonate</td>
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<td>MnSO₄</td>
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<td>0.012</td>
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<tr>
<td>Calcium lactate</td>
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</tr>
<tr>
<td>DHA source oil⁶</td>
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<tr>
<td>Soy lecithin</td>
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</table>

Calculated composition (%)

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<th>Ingredient</th>
<th>Control</th>
<th>AA/DHA–enriched</th>
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<td>Crude protein</td>
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<tr>
<td>Lactose</td>
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<td>33.0607</td>
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<tr>
<td>Fat</td>
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<td>25.716</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.9063</td>
<td>0.9063</td>
</tr>
<tr>
<td>Phosphorus available</td>
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<td>0.70</td>
</tr>
<tr>
<td>Metabolizable energy (MJ/kg)</td>
<td>20.27</td>
<td>20.27</td>
</tr>
</tbody>
</table>

1 AA/DHA, arachidonic acid and docosahexaenoic acid; AIN-93, American Institute of Nutrition–93.
2 The ratio of casein to whey is approximately 1:1.
3 Supplied per kg of dry formula: 3 g nicotinic acid, 1.6 g calcium pantotenate, 0.7 g pyridoxine HCl, 0.6 g thiamine HCl, 0.6 g riboflavin, 0.2 g folic acid, 0.02 g d-biotin, 2.5 g vitamin B-12 (0.1% in mannitol), 15 g L-γ-tocopherol acetate (500 IU/g), 0.8 g vitamin A palmitate (500 000 IU/g), 0.2 g vitamin D₃ (cholecalciferol, 500 000 IU/g), 0.075 g vitamin K (phyloquinone), and 974.705 g sucrose.
4 The sow-milk replacer AA/DHA–enriched fat blend contained 44% palm olein oil, 20% soybean oil, 20% coconut oil, and 14.88% high-oleic sunflower oil.
5 The source of AA contained 40% AA; the balance was palmitic acid (5–15%), stearic acid (10–20%), oleic acid (12–39%), linoleic acid (5–15%), and linolenic acid (2.5–5%).
6 The source of DHA contained 40% DHA; the balance was myristic acid (10–20%), palmitic acid (15–20%), oleic acid (10–30%), and linoleic acid (1–5%).

Proliferation assays

The proliferation assays were performed on PBMCs isolated from all of the piglets on days 0, 9 (before the vaccination), 16, 23, and 30 after weaning. To measure overall lymphocyte proliferation, we used a lymphocyte blastogenesis test based on the incorporation of titrated thymidine. Briefly, flat-bottomed, 96-well microtiter plates (Falcon 3072; Becton Dickinson, Lincoln Park, NJ) were seeded with 100 μL PBMCs at 2 × 10⁶ cells/mL and 100 μL of media alone (nonstimulated wells), media containing the influenza virus antigens (20 μg/mL), or media containing concanavalin A (ConA; 5 μg/mL) as an internal proliferation control. Preliminary validation assays using ConA or influenza antigen at 2.5, 5, 10, and 20 μg/mL were conducted to assess the optimal lymphocyte stimulation under the current experimental conditions and within the genetic background and age of the pigs. Plates were incubated for 5 d at 37 °C in a 5% CO₂ humidified atmosphere. After 5 d of culture, 0.5 μCi methyl-[³H] thymidine (specific radioactivity: 6.7 Ci mmol−¹; Amersham Life Science, Arlington Heights, IL) in 10 μL medium was added to each well, and the plates were incubated for an additional 20 h. Twenty hours after thymidine addition, cells were harvested onto glass fiber filters with a Combicell harvester (Skatron Instruments, Sterling, VA), and incorporated radioactivity was measured by liquid scintillation counting (LS 6500; Beckman Instruments, Palo Alto, CA). Overall lymphocyte proliferation results were expressed as stimulation indexes, which were calculated by dividing the counts per minute of antigen-stimulated wells by the counts per minute of nonstimulated wells.

To determine subset-specific proliferation, PBMCs were labeled with carboxyfluorescein diacetate, succinimidy ester (CFSE; Molecular Probes, Eugene, OR), a fluorescent dye used for tracking cell division. CFSE is an ester that diffuses into cells, where it reacts with amine groups, becoming fluorescent. The label is stably retained by proteins and, after cell division, it is equally distributed between daughter cell populations. Briefly, 10⁶ PBMCs were incubated for 15 min at room temperature in 1 mL complete RPMI containing 1 μmol CFSE/L. After 2 extra washes, cells were resuspended in complete RPMI and enumerated, and the cell concentration was normalized to 25 × 10⁶/mL. Cells were cultured with media alone or stimulated with influenza virus antigens or ConA. Cells were harvested on day 5, diluted 1:4 in phosphate-buffered saline (PBS). Mononuclear cells located in the interface between the diluted plasma and the lymphoprep were recovered by using a sterile Pasteur pipette. PBMCs were washed twice with PBS and resuspended in complete medium. Complete medium was prepared by supplementing RPMI-1640 with 25 mmol HEPES buffer/L (Sigma, St. Louis, MI), 100 units penicillin/mL (Sigma), 0.1 mg streptomycin/mL (Sigma), 5 × 10⁻⁵ mol 2-mercaptoethanol/L (Sigma), 1 mmol essential amino acids/L (Mediatech), 1 mmol nonessential amino acids/L (Sigma), 2 mmol l-glutamine/L (Sigma), 1 mmol sodium pyruvate/L (Sigma) and 10% fetal bovine serum. Media pH was measured with a pH meter (Orion Research Inc, Beverly, MA) and adjusted to 7.4 with addition of a solution of 7.5% sodium bicarbonate (Fisher Scientific, Pittsburgh, PA). The remaining peripheral blood was used to determine the total white blood cell counts by using a single-particle counter (Coulter Z1; Beckman Coulter Corp, Miami, FL). Differential counts were performed by using flow cytometry based on forward and side scatter after the lysis of red blood cells.

Harvesting of peripheral blood mononuclear cells

On days 0, 9, 16, 23, and 30 of the study, whole blood was obtained by vena cava puncture with sterile, heparinized, 10-mL evacuated tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by using a previously described gradient centrifugation procedure (21). Briefly, PBMCs were isolated by overlaying lymphoprep (Mediatech, Herndon, VA) with whole blood
seeded in 96 round-bottom microtiter plates, and stained with anti-pig CD4 (clone 74–12–4), anti-pig CD8a (clone 76–2–11), anti-pig-CD8β (clone PG164A), anti-pig-IgM (clone PG145A), and appropriate secondary antibody combinations. Data acquisition was performed in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

**Immunophenotyping of peripheral blood mononuclear cells**

PBMCs were labeled with anti-pig primary antibodies as previously described (21). The primary antibodies were phycoerythrin-labeled anti-pig-CD4 (clone 74–12–4), biotinylated immunoglobulin G (IgG)2a mouse anti-pig-CD8α (clone 76–2–11) (22), IgG2a mouse anti-pig-CD8β (clone PG164A), IgG1 mouse anti-pig-CD45RA (clone PG96A), IgG2b mouse anti-pig-SWC3 (clone 74–22–15), and IgM mouse anti-pig-IgM (clone PG145A) (all: VMRD Inc, Pullman, WA), and appropriate secondary antibody combinations (Southern Biotechnology Associates, Birmingham, AL). Flow cytometric data acquisition and analyses were performed as previously described (21, 23).

**Necropsy procedures and tissue collection and storage**

During the course of the experiment, we monitored piglets for signs of hypothermia, lethargy, and enteric disease according to a disease activity index. On day 30 of the study, piglets were euthanized by an intravenous injection of Sleepaway (Fort Dodge Laboratories, Fort Dodge, IA), a sodium pentobarbital solution. Then, the mediastinal lymph nodes were collected in sterile conditions. All organs (including the heart, lungs, thymus, brain, liver, stomach, pancreas, spleen, kidneys, duodenum, jejunum/ileum, and colon) were excised and weighed, and samples were snap-frozen in liquid nitrogen for subsequent storage at −80 °C. Ileal samples were also collected in tissue freezing medium in cryomolds and frozen at −80 °C for immunohistochemical evaluation of mucosal secretory IgA and in RNAlater solution (Ambion, Austin, TX) for RNA isolation and gene expression analyses. Samples of spleen, thymus, ileum, and colon were also fixed in a phosphate-buffered neutral 10% formalin solution for subsequent histologic analyses.

**Delayed-type hypersensitivity reaction for influenza virus antigens**

DTH represents a widely accepted in vivo measurement of cell-mediated immunity. The current study examined the effects of infant formula and influenza virus vaccination on the induction of cell-mediated, influenza virus–specific responses in vivo. Briefly, on day 28 of the study, the ear was disinfected with a 70% ethanol solution. All pigs received an intradermal injection of influenza virus antigen (100 μg in 150 μL) or PBS (negative control for nonspecific inflammation due to physical irritation or injection) at different locations on the dorsal aspect of the right ear. Injection sites were examined at 0 and 24 h for erythema, edema, and induration. In addition, induration was measured by using calipers and recorded in millimeters. The percentage increase in skin thickness was calculated as follows: [(vaccine antigen 24 h – vaccine antigen 0 h)/vaccine antigen 0 h] – [(PBS 24 h – PBS 0 h)/PBS 0 h]. DTH to influenza virus antigens has previously been used to investigate the immunomodulatory actions of nutrients in vivo (24).

**Real-time, quantitative reverse transcriptase–polymerase chain reaction**

For RNA isolation, PBMCs and spleen samples were stored in RNAlater (Ambion), and total RNA was isolated by using the Mini RNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Total RNA (1 μg) from each sample was used to generate complementary DNA (cDNA) template by using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The total reaction volume was 20 μL. The reaction was incubated as follows in an iCycler iQ thermal cycler (Bio-Rad): 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C, and hold at 4 °C. Controls were also performed with no RNA template (no template) and by omitting the reverse transcriptase enzyme (no RT). The PCR primer pairs were designed, on the basis of sequences previously published by GeneBank, with the use of the Oligo 6 primer design software (version 5; Molecular Biology Insights, Cascade, CO), and predicted amplicon sequences were checked online by using a BLAST search. The PCR primer pair sequences, annealing temperatures, accession numbers, and PCR product lengths are outlined in Table 2.

PCR was performed on the cDNA by using the iScript cDNA Kit (Bio-Rad) under previously described conditions (19, 25); each gene amplicon was purified by using the MiniElute PCR Purification kit (Qiagen). The purified amplicon for each gene was quantitated on an agarose gel by using a DNA mass ladder. The purified amplicons were further used to optimize the real-time PCR conditions and to generate the standard curves in the real-time PCR assay. Primer concentrations and annealing temperatures were optimized for the optics module of the iCycler iQ thermal cycler for each set of primers by using the system’s gradient protocol. PCR efficiencies were maintained at 92–105% for each primer set during optimization and also during the real-time PCR of sample cDNA. Messenger RNA (mRNA) expression of cytokines was measured with real-time, quantitative PCR by using an iCycler iQ System and the iQ SYBR Green Supermix fluorescent dye (Bio-Rad). Real-time PCR was used to measure the starting amount of nucleic acid by assaying each unknown starting quantity of cDNA on a 96-well plate. For each gene, the results were calculated as the starting quantity of target cDNA (in pg) per microgram of colonic RNA. Because the expression of the housekeeping gene (ie, β-actin) differed significantly between treatment groups, data are presented as picograms of cDNA for each gene of interest divided by the picograms of β-actin.

**Statistical analysis**

Analysis of variance (ANOVA) was used to determine the main effects of the dietary treatment or the immunization status and the interaction between dietary treatment and immunization status. ANOVA was performed by using the general linear model procedure in SAS software (version 9.1.3) (26) as previously described (19, 25). P < 0.05 was considered to be significant. Before the influenza virus immunizations, data were analyzed as a repeated-measures randomized complete block design. For data collected at the end of the study as a single timepoint measurement, postimmunization data were analyzed as a 2 × 3 factorial arrangement of treatments within a split-plot design. In the model, 3 pigs within 1 block was the experimental unit for the dietary treatment (subplot), and the blocks of pigs within each
immunization status were the experimental units for immunization treatment (whole plot). The whole-plot error (ie, error A) was block within immunization status (ie, 7 df), and the subplot error (ie, error B) was the residual df after accounting for the dietary treatment variance and the variance for the interaction between dietary treatment and infective status (ie, 35 df). The statistical model utilized was given in the following equation:

\[ Y_{ijk} = \mu + \text{immunization}_i + \text{error } A_{ik} + \text{diet}_j + (\text{immunization} \times \text{diet})_{ij} + \text{error } B_{ijk} \]  

where \( \mu \) was the general mean, Immunization was the main effect of the \( i \)th level of the vaccine effect, Diet was the main effect of the \( j \)th level of the dietary effect, (Immunization \times Diet) was the interaction effect between immunization and diet, and errors A and B represented the random errors for the whole plot and the subplot, respectively.

For analyzing the measurements on the kinetics of the immune response and body weight data over time, we used a 3-factor repeated-measures ANOVA. For this analysis, in addition to the main effects of diet, vaccine, and the 2-factor interaction between diet and immunization (as shown above), the model included the main effect of time, the diet \( \times \) time and immunization \( \times \) time interactions, and the 3-factor interaction (diet \( \times \) immunization \( \times \) time). The ANOVA was followed by Sheffe’s post hoc multiple comparison analyses.

RESULTS

Effect of an arachidonic acid– and docosahexaenoic acid–enriched formula on body weight and organ weight

Body weight and formula intake were measured daily to determine the effect of AA/DHA-enriched formula on growth and appetite. All piglets within a block were very similar (ie, sex, litter, and initial body weight) on day 0 of the study. No significant differences were found in body weight between treatment groups during the first 2 d of the study. However, the sow-reared piglets grew significantly faster than did piglets fed the control or AA/DHA-enriched formulas between days 3 and 15 of the study (Figure 1). The influenza virus vaccination had no effect on piglet body weight throughout the study (Figure 1B and C).

When piglets fed the control and the AA/DHA-supplemented formulas were compared, the daily intake of formula did not differ significantly between groups throughout the course of the study except days 22 and 26, when formula intake was greater in piglets fed the control formula than in those fed the supplemented formulas (data not shown). To limit the effect of additional handling on the immunologic endpoints, the intake of sow milk in sow-reared piglets was not measured. Sow-reared piglets had significantly smaller organs, including liver, spleen, kidneys, jejunum or ileum, and colon, than did formula-fed piglets, but no significant differences in organ weight were found between piglets fed the control formula and those fed the AA/DHA-supplemented formula (Table 3). However, the brain was significantly larger in sow-reared piglets than in formula-fed piglets, regardless of AA/DHA supplementation (Table 3). No significant differences in enteric clinical activity were found between treatment groups during the entire study (data not shown).

Effect of the formulas and sow milk on T cell subsets in piglet peripheral blood

The lymphocyte subpopulations in peripheral blood of piglets are functionally and phenotypically heterogeneous. A brief summary of the distinct T and B cell subsets identified in pigs is included in the Discussion. No significant differences in the numbers of peripheral blood CD4\(^+\), CD8\(\alpha^+\), or CD4\(^+\)CD8\(\alpha^+\) T cells were found between groups on days 0 and 16 of the study. On day 9, the numbers of CD8\(\alpha^+\) cells were significantly greater in piglets fed the control formula than in sow-reared piglets or those fed AA/DHA-enriched formula (Table 4). On day 30, the numbers of peripheral blood CD8\(\alpha^+\) cells were lower in sow-reared piglets than in the formula-fed groups (Table 4). The

<table>
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<th>Primer sets</th>
<th>Sequences (5’ to 3’)</th>
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<td>TNF-αR</td>
<td>TTAATTTCTGCCACTGAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10F</td>
<td>CGCCTAACCCTAGGAGAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10R</td>
<td>GCCACGAGTTCTCAAGAA</td>
<td>70</td>
<td>NM.214015</td>
</tr>
<tr>
<td>TGF-β1F</td>
<td>CTTCCCAAGCCACAGGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β1R</td>
<td>CGGCCAGGGGGTCAGAATGT</td>
<td>104</td>
<td>AB194009</td>
</tr>
<tr>
<td>IL-2F</td>
<td>CTCTGGAGGGAGTCTCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2R</td>
<td>TCCCTTTACTTCCAAAACTG</td>
<td>115</td>
<td>U07786</td>
</tr>
<tr>
<td>β-actinF</td>
<td>CTCTTCAGCCTTCCTTCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actinR</td>
<td>GCACCGTGTGGCGTAGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) IFN, interferon; IL, interleukin; TNF-α, tumor necrosis factor α; TGF, transforming growth factor; F, forward; R, reverse. PCR primer pairs were designed for an optimal annealing temperature between 50 and 60°C and product length between 70 and 150 base pairs. When plotting threshold cycle versus log starting cDNA quantity (pg), standard curves had slopes between −3.2 and −3.5; polymerase chain reaction efficiencies were between 92% and 105% and \( R^2 \) was above 0.98.
numbers of CD4+ T cells were transiently higher on day 23 in sow-reared piglets than in the other diet groups (Table 4). The distribution of memory (CD45RC lowCD45RA low) and naïve (CD45RChighCD45RAhigh) CD8+/+H-9251/H-9252/L-1151 T cells, as measured by CD45RC and CD45RA expression, remained unchanged from day 0 to day 23 of the study (Table 5). However, on day 30, the numbers of CD8αβ+CD45RC highCD45RA low naïve PBMCs were significantly lower in sow-reared piglets than in the other 2 diet groups (Table 5). Also on day 30, the CD8αβ+CD45RC highCD45RA low T cell numbers were significantly lower in vaccinated piglets, regardless of diet, than in unvaccinated piglets fed the control formula (Table 5). These cells may correspond to an activated phenotype that is highly differentiated by exposure to antigen in vivo (27).

**Nutritional modulation of antigen-specific proliferation of peripheral blood mononuclear cells**

We measured overall lymphoproliferation induced by influenza virus antigens or the mitogen ConA ex vivo by using a lymphocyte blastogenesis test (LBT). A 3-factor interaction among diet, immunization, and time was significant ($P < 0.001$) for the virus-induced but not for the ConA-induced lymphoproliferation. As anticipated, no influenza virus–specific responses were detected before (ie, days 0 and 9) or after immunization in nonvaccinated piglets throughout the study (Table 6). On day 16, the influenza virus–specific proliferation of PBMCs recovered from unvaccinated piglets fed the control formula was lower than that of PBMCs from vaccinated piglets fed the AA/DHA-enriched formula. In addition, on day 23, the lymphocytes recovered from vaccinated piglets fed the control formula had a significantly greater influenza virus–specific proliferative ability than did the lymphocytes recovered from all unvaccinated piglet groups or vaccinated sow-reared piglets. In comparison with the proliferative ability of lymphocytes recovered from vaccinated piglets fed the control formula, that of lymphocytes from piglets fed AA/DHA-enriched formula was numerically (virus stimulation index of 138.37 and 36.38, respectively) but not significantly lower (Table 6).

**FIGURE 1.** Least-squares mean (±SEM) body weights before (days 0 –9; A) and after (days 10 –30) immunization treatment with an influenza virus vaccine in nonimmunized (B) and immunized (C) piglets fed control formula (○), arachidonic acid– and docosahexaenoic acid– (AA/DHA; ♣) enriched formula, or sow milk (●). Piglets were immunized with an inactivated influenza virus vaccine on day 9 of the experiment. $n = 16$ from day 0 to day 9; $n = 8$ from day 10 to day 30. The error bars at younger ages are smaller than the symbols and cannot be seen in the figure. Before the immunization treatment, data were analyzed as a repeated-measures randomized complete-block design. After the immunization treatment, data were analyzed as a repeated-measures factorial arrangement within a split-plot design. Immunization status represents the whole plot, and dietary treatments represent the subplot. The experimental unit for the whole plot was a block of 3 littermate piglets, and that for the subplot was 3 piglets within 1 block. The 3-factor interaction (diet × immunization × time) was not significant. However, the 2-factor diet × time interaction was significant ($P < 0.0001$). Scheffe’s multiple-comparisons test was used after ANOVA. * Significant differences among treatments attributed to the effects of diet over time, $P < 0.05$. **Nutritional modulation of antigen-specific proliferation of T cell subsets**

To measure subset-specific proliferation, we used the CFSE proliferation assay. In contrast to the LBT, which measures only lymphoproliferation occurring after the addition of titrated thymidine (20-h proliferation), the CFSE assay measures cumulative proliferation over the 5-d culture. Thus, it is a more sensitive...
TABLE 3
Effect of dietary treatments on organ weight adjusted by metabolic body weight (BW<sup>0.75</sup>) in neonatal piglets fed control, arachidonic acid– (AA) and docosahexaenoic acid– (DHA) enriched formula, or sow milk after immunization with an influenza virus vaccine<sup>1</sup>

<table>
<thead>
<tr>
<th>Organ</th>
<th>Nonvaccinated pigs</th>
<th>Vaccinated pigs&lt;sup&gt;2&lt;/sup&gt;</th>
<th>P&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control AA/DHA Sow</td>
<td>Control AA/DHA Sow</td>
<td></td>
</tr>
<tr>
<td></td>
<td>g/BW&lt;sup&gt;0.75&lt;/sup&gt;</td>
<td>g/BW&lt;sup&gt;0.75&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>7.86 7.71 7.85</td>
<td>6.72 7.27 7.81</td>
<td>0.51 NS</td>
</tr>
<tr>
<td>Lungs</td>
<td>13.63 13.20 13.44</td>
<td>12.87 12.28 11.68 2.56</td>
<td>NS NS</td>
</tr>
<tr>
<td>Thymus</td>
<td>2.07 2.36 2.41</td>
<td>2.89 3.30 2.48 0.37</td>
<td>NS 0.02</td>
</tr>
<tr>
<td>Brain</td>
<td>4.50 4.95 5.40</td>
<td>4.70 4.92 5.52 0.20</td>
<td>0.0002&lt;sup&gt;4&lt;/sup&gt; NS</td>
</tr>
<tr>
<td>Liver</td>
<td>31.27 33.12 21.26</td>
<td>30.60 30.45 22.70 1.20</td>
<td>0.0001&lt;sup&gt;4&lt;/sup&gt; NS</td>
</tr>
<tr>
<td>Stomach</td>
<td>5.84 5.44 5.27</td>
<td>5.50 5.55 4.89 0.28</td>
<td>NS NS</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1.17 1.33 1.12</td>
<td>1.50 1.43 1.17 0.12</td>
<td>NS NS</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.27 3.86 2.34</td>
<td>4.54 4.34 3.21 0.37</td>
<td>0.0006&lt;sup&gt;4&lt;/sup&gt; 0.002</td>
</tr>
<tr>
<td>Kidneys</td>
<td>6.97 7.66 5.43</td>
<td>7.11 7.07 5.01 0.35</td>
<td>0.0001&lt;sup&gt;4&lt;/sup&gt; NS</td>
</tr>
<tr>
<td>Duodenum</td>
<td>1.93 2.30 1.58</td>
<td>1.74 1.47 1.57 0.29</td>
<td>NS NS</td>
</tr>
<tr>
<td>Jejunum or ileum</td>
<td>50.52 49.26 26.09</td>
<td>45.67 46.44 26.61 2.30</td>
<td>0.0001&lt;sup&gt;4&lt;/sup&gt; NS</td>
</tr>
<tr>
<td>Colon</td>
<td>20.78 19.27 11.50</td>
<td>19.29 20.80 12.70 2.08</td>
<td>0.001&lt;sup&gt;4&lt;/sup&gt; NS</td>
</tr>
</tbody>
</table>

<sup>1</sup>D, the main effect of the diet; V, the main effect of vaccination. Data are presented as least-squares $\bar{x}$ (n = 8; day 30) for a particular organ weight.

<sup>2</sup>On day 9, 8 blocks of 3 pigs each were vaccinated as described in Materials and Methods.

<sup>3</sup>Before vaccination (eg, on days 0 and 9), data were analyzed as a randomized complete-block design. After vaccination, data were analyzed as a 2 × 3 factorial arrangement (ie, 2 vaccination status and 3 dietary treatments) within a split-plot design. Vaccination status represents the whole plot and dietary treatments represent the subplot. The experimental unit for the whole plot was a block of 3 litters per piglet, and that for the subplot was 3 piglets within a block. Analysis was conducted by ANOVA. The interaction between vaccination status and dietary treatments was not significant, and, therefore, it is not reported. Because the interaction was not significant, comparisons are not reported across all 6 means in a row.

<sup>4</sup>Multiple comparison analyses were conducted only to determine differences among diet groups. Post hoc testing indicated that the adjusted organ weights for the sow-suckled piglets were significantly different ($P < 0.05$) from those for the formula-fed groups. Scheffe’s multiple-comparisons test was used after ANOVA.

assay. In addition, by coupling the CFSE assay with staining for cell surface markers, we measured the proliferation of distinct lymphocyte subsets. The flow cytometric evaluation of the CFSE-stained T cell subsets found that CD4<sup>+</sup> T cell proliferation followed a pattern resembling that of overall lymphocyte influenza virus–specific proliferation, as measured by LBT. Specifically, on days 16, 23, and 30, a main effect of vaccination was observed in vaccinated piglets. The 2-factor immunization × time ($P < 0.001$) and immunization × diet ($P < 0.04$) interactions were significant for virus-specific CD4<sup>+</sup> T cell proliferation; the 2-factor diet × time ($P < 0.01$) and immunization × time ($P < 0.0001$) interactions were significant for CD8αα<sup>+</sup> T cell proliferation; and the 3-factor interaction (immunization × diet × time) was significant ($P < 0.002$) for CD8αβ<sup>+</sup> T cell proliferation. Also on day 23, CD4<sup>+</sup>, CD8αα<sup>+</sup>, and CD8αβ<sup>+</sup> T cells recovered from piglets fed the control formula had significantly greater proliferative responses to ex vivo influenza virus stimulation than did cells from the other treatment groups (Figures 2, 3, and 4). By day 30 of the study, the magnitude of postimmunization cell-mediated responses had decreased to close to basal levels, and no significant effects of diet were observed on influenza virus–specific T cell responses (Figures 2–4). Thus, antigen-specific T cell responses were detectable on day 16 (day 7 after immunization), peaked on day 23 (day 14 after immunization), and returned to close to baseline by day 30 (day 21 after immunization).

**Delayed-type hypersensitivity reaction**

On day 28, we administered a DTH response test in the right ear to measure influenza virus–specific cell-mediated immune responses in vivo. We found a highly significant effect of vaccination on DTH values measured as percentage increase of skin induration. Even though the DTH values in vaccinated piglets fed the control formula were numerically greater than those in the other 5 treatment groups, this numerical difference was not statistically significant (Figure 5). Thus, the results of in vivo cell-mediated immunity are in line with and validate the results of ex vivo T cell lymphoproliferative responses to influenza virus stimulation.

**Effect of arachidonic acid– and docosahexaenoic acid–enriched formula on cytokine expression of peripheral blood mononuclear cells**

Proliferation and effector functions of T cells are controlled through both cell contact–dependent and soluble cytokine–dependent mechanisms. In the current study, we examined cytokine mRNA expression [ie, IL-10, transforming growth factor β1 (TGF-β1), IL-2, IL-13, interferon γ (INF-γ), tumor necrosis factor α (TNF-α), and IL-4] in spleen and PBMCs. We found that the expression of the antiinflammatory and immunoregulatory cytokine IL-10 was significantly greater in piglets fed the AA/DHA–enriched formulas or sow milk than in those fed the control formula (Figure 6). No significant differences in expression of TGF-β1, IL-2, IL-13, IFN-γ, TNF-α, or IL-4 were found between dietary treatment groups (data not shown).

**DISCUSSION**

Nutritional immunology studies have revealed important roles for dietary PUFAs, including AA and DHA, in the modulation of innate and adaptive immune responses (12, 13, 21, 23, 28,
29). Immunization and challenge studies have been instrumental in dissecting the immunomodulatory properties and mechanisms of immune modulation of dietary components in general and fatty acids in particular. For example, we found that, after immunization with viral antigens, CLA enhanced effector functions of CD8+ T cells that are critical for the clearance of viruses—ie, antigen-specific proliferation and granzyme activity in adult pigs (23). Kelley et al (12) reported that dietary AA supplementation enhanced postimmunization influenza virus–specific lymphoproliferation in adult humans. However, little is known about the immunomodulatory properties of AA and DHA when they are fed in combination, as during the early neonatal period. In this study, antigen-specific T cell responses were the primary endpoint criterion, whereas growth, feed intake, organ weight, and cytokine expression were secondary endpoints.

We found that the body weight and formula intake did not differ significantly between the piglets fed the control formula and those fed the AA/DHA-enriched formula. Thus, immunologic differences between these 2 groups could not be explained on the basis of growth- or energy intake–related differences. These results with respect to body weight are consistent with previous reports indicating that the detrimental effects of DHA in growth can be overcome by feeding it in combination with AA (30, 31). The inclusion of a third group of sow-reared piglets was intended to provide a means of comparing the 2 experimental formulas with a reference group resembling breastfeeding. In this regard, infant formula is defined by law as a food for use in infants that simulates human milk or that is suitable as a complete or partial substitute of human milk. In line with the greater body weight and length associated with breastfeeding, we found that sow-reared piglets grew at a faster rate from days 3 to 15 of the study, which can be explained by the fact that the energy content of sow milk during the initial days of lactation is greater than that of the experimental formulas used in the current study (1363 and 970 kcal/kg, respectively) (32).

### Table 4

<table>
<thead>
<tr>
<th></th>
<th>Nonvaccinated pigs</th>
<th>Vaccinated pigs</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>AA/DHA</td>
<td>Sow</td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>1.15</td>
<td>1.30</td>
<td>1.16</td>
</tr>
<tr>
<td>CD4CD8α</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>CD8α</td>
<td>0.38</td>
<td>0.42</td>
<td>0.35</td>
</tr>
<tr>
<td>Day 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>2.15</td>
<td>2.02</td>
<td>2.42</td>
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<tr>
<td>CD4CD8α</td>
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<td>0.09</td>
</tr>
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<td>CD8α</td>
<td>1.48a</td>
<td>1.05b</td>
<td>1.02b</td>
</tr>
<tr>
<td>Day 16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>1.66</td>
<td>1.79</td>
<td>2.56</td>
</tr>
<tr>
<td>CD4CD8α</td>
<td>0.15</td>
<td>0.14</td>
<td>0.13</td>
</tr>
<tr>
<td>CD8α</td>
<td>1.42</td>
<td>1.25</td>
<td>1.29</td>
</tr>
<tr>
<td>Day 23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>1.34</td>
<td>1.32</td>
<td>2.18</td>
</tr>
<tr>
<td>CD4CD8α</td>
<td>0.23</td>
<td>0.13</td>
<td>0.22</td>
</tr>
<tr>
<td>CD8α</td>
<td>1.50</td>
<td>1.50</td>
<td>1.45</td>
</tr>
<tr>
<td>Day 30</td>
<td></td>
<td></td>
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<tr>
<td>CD4</td>
<td>1.83</td>
<td>1.61</td>
<td>1.59</td>
</tr>
<tr>
<td>CD4CD8α</td>
<td>0.41</td>
<td>0.32</td>
<td>0.19</td>
</tr>
<tr>
<td>CD8α</td>
<td>2.83</td>
<td>1.91</td>
<td>0.92</td>
</tr>
</tbody>
</table>

1. D, the main effect of the diet; V, the main effect of vaccination. Least-squares mean values (n = 16, days 0–9; n = 8, days 10–30). Values in a row with different superscript letters are significantly different, P < 0.05.

2. On day 9, 8 blocks of 3 pigs each were vaccinated as described in Materials and Methods.

3. Before vaccination (eg, days 0 and 9), data were analyzed as a repeated-measures randomized complete-block design. After vaccination, data were analyzed as a repeated-measures factorial arrangement within a split-plot design (both analyses used ANOVA). Vaccination status represents the whole plot, and dietary treatments represent the subplot. The experimental unit for the whole plot was a block of 3 littermate pigs, and that for the subplot was 3 piglets within 1 block. The 3-factor interaction (vaccination × diet × time) or the 2-factor interaction (vaccination × diet) were not significant for any of the 3 markers examined and are not shown in the table. The diet × time interaction was significant for CD8α (P < 0.0002) and CD4CD8α (P < 0.05), and the vaccine × time interaction was significant for CD4 (P < 0.004).

4. Includes the T cell subsets TCRγCD8α, CD3+CD16+CD8α (natural killer cells), and TCRαβCD8αβ.

5. Multiple comparison analyses were conducted to determine differences among diet groups. Post hoc testing indicated that the numbers of CD4+ T cells for the sow-suckled piglets were significantly different (P < 0.05) from those for the formula-fed groups.

6. Multiple comparison analyses were conducted to determine differences among diet groups. Post hoc testing indicated that the numbers of CD8α+ T cells for the sow-suckled piglets were significantly different (P < 0.05) from those for the control formula-fed group. Scheffé’s multiple-comparisons test was used after ANOVA.
Specifically, the response was detectable on day 16 (ie, day 7 after immunization), peaked on day 23 (ie, day 14 after immunization), and returned to nearly baseline on day 30 (ie, day 21 after immunization). On day 23, the proliferative abilities of CD4⁺ T cells to influenza virus stimulation were significantly greater in immunized piglets fed the control formula than in immunized piglets fed AA/DHA-enriched formula or sow-reared vaccinated piglets. Thus, it appears that the AA/DHA-enriched formula and sow milk may have suppressed influenza virus–specific T cell responses on day 23 after feeding.

Porcine CD8⁺ lymphocytes can be subdivided phenotypically into 5 subpopulations on the basis of T cell receptor (TCR) and coreceptor expression: 1) CD8α⁺CD4⁻; 2) CD8α⁺CD4⁺; 3) CD8α⁺CD3⁻CD16⁺ (a subset of natural killer cells); 4) CD8α⁺TCRα⁺CD4⁺ (potentially immunoregulatory); and 5) CD8α⁺TCRα⁺CD4⁻ (cytotoxic) (21). Conversely, the porcine CD4⁺ T cell population is more homogeneous and can be subdivided into 2 populations: 1) CD8α⁺TCRα⁺ and 2) CD8α⁺TCRα⁻ (33). CD4⁺ CD8α⁺ T cell responses were consistent with important numerical differences between groups in the DTH reaction test, an in vivo assessment of cell mediated immunity, although the numerical differences in the DTH response were not statistically significant. It is interesting that the DTH response observed in the AA/DHA-enriched group was similar to that observed in the sow milk–fed group.

TABLE 5
Effect of dietary treatments on total numbers of CD8αβCD45RC⁺CD45RA⁻, CD8αβCD45RC⁺CD45RA⁺, CD8αβCD45RC⁺CD45RA⁻, and CD8αβCD45RC⁺CD45RA⁻ peripheral blood lymphocytes in neonatal piglets fed control, arachidonic acid (AA) and docosahexaenoic acid (DHA) enriched formula, or sow milk before and after immunization with an influenza virus vaccine¹

<table>
<thead>
<tr>
<th></th>
<th>Nonvaccinated pigs</th>
<th>Vaccinated pigs²</th>
<th>P⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control AA/DHA Sow</td>
<td>Control AA/DHA Sow</td>
<td>SEM</td>
</tr>
<tr>
<td>Day 0</td>
<td>10⁶ cells/mL blood</td>
<td>10⁶ cells/mL blood</td>
<td></td>
</tr>
<tr>
<td>CD45RC⁺CD45RA⁻</td>
<td>0.081 0.054 0.057</td>
<td>—     —     —</td>
<td>0.01</td>
</tr>
<tr>
<td>CD45RC⁺CD45RA⁻</td>
<td>0.151 0.153 0.148</td>
<td>—     —     —</td>
<td>0.02</td>
</tr>
<tr>
<td>CD45RC⁺CD45RA⁻</td>
<td>0.187 0.107 0.109</td>
<td>—     —     —</td>
<td>0.04</td>
</tr>
<tr>
<td>CD45RC⁺CD45RA⁻</td>
<td>0.043 0.057 0.052</td>
<td>—     —     —</td>
<td>0.005</td>
</tr>
<tr>
<td>Day 9</td>
<td>10⁶ cells/mL blood</td>
<td>10⁶ cells/mL blood</td>
<td></td>
</tr>
<tr>
<td>CD45RC⁺CD45RA⁻</td>
<td>0.093 0.062 0.086</td>
<td>—     —     —</td>
<td>0.01</td>
</tr>
<tr>
<td>CD45RC⁺CD45RA⁻</td>
<td>0.174 0.203 0.261</td>
<td>—     —     —</td>
<td>0.03</td>
</tr>
<tr>
<td>CD45RC⁺CD45RA⁻</td>
<td>0.075 0.068 0.070</td>
<td>—     —     —</td>
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</tr>
<tr>
<td>CD45RC⁺CD45RA⁻</td>
<td>0.183 0.138 0.164</td>
<td>—     —     —</td>
<td>0.02</td>
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<tr>
<td>Day 16</td>
<td>10⁶ cells/mL blood</td>
<td>10⁶ cells/mL blood</td>
<td></td>
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<tr>
<td>CD45RC⁺CD45RA⁻</td>
<td>0.086 0.089 0.129</td>
<td>0.111 0.145 0.131</td>
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<tr>
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<td>CD45RC⁺CD45RA⁻</td>
<td>0.313 0.265 0.210</td>
<td>0.258 0.223 0.250</td>
<td>0.04</td>
</tr>
<tr>
<td>Day 23</td>
<td>10⁶ cells/mL blood</td>
<td>10⁶ cells/mL blood</td>
<td></td>
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<td>CD45RC⁺CD45RA⁻</td>
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<td>0.107 0.066 0.169</td>
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<td>CD45RC⁺CD45RA⁻</td>
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<td>0.030 0.033 0.037</td>
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<tr>
<td>Day 30</td>
<td>10⁶ cells/mL blood</td>
<td>10⁶ cells/mL blood</td>
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<tr>
<td>CD45RC⁺CD45RA⁻</td>
<td>0.295⁺ 0.136⁺ 0.146⁺</td>
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<td>CD45RC⁺CD45RA⁻</td>
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<td>0.05</td>
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<td>CD45RC⁺CD45RA⁻</td>
<td>0.243 0.209 0.127</td>
<td>0.581 0.671 0.129</td>
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¹ D, the main effect of the diet; V, the main effect of vaccination; D × V represents the interaction between the vaccination status and the dietary treatments; hi, high; lo, low. Least-squares mean values (n = 16, days 0–9; n = 8, days 10–30). Values in a row with different superscript letters are significantly different, P < 0.05.

² On day 9, 8 blocks of 3 pigs each were vaccinated as described in Materials and Methods.

³ Before vaccination (eg, days 0 and 9), data were analyzed as a repeated-measures randomized complete-block design. After vaccination, data were analyzed as a repeated-measures factorial arrangement within a split-plot design (both analyses used ANOVA). Vaccination status represents the whole plot, and dietary treatments represent the subplot. The experimental unit for the whole plot was a block of 3 littermate pigs, and that for the subplot was 3 piglets within 1 block. The 3-factor interaction (vaccination × diet × time) was not significant for CD45RC⁺CD45RA⁻, CD45RC⁺CD45RA⁻, CD45RC⁺CD45RA⁻, or CD45RC⁺CD45RA⁻. The 2-factor D × V interaction was significant for CD45RC⁺CD45RA⁻ (P < 0.01) but not for the other 3 subsets. The 2-factor vaccine × time interaction was significant for CD45RC⁺CD45RA⁻ (P < 0.01) but not for the other 3 subsets.

⁴ Multiple comparisons analyses were conducted only to determine differences among diet groups. Post hoc testing indicated that the numbers of CD45RC⁺CD45RA⁻ in sow-suckled piglets was significantly different (P < 0.05) from those in formula-fed groups. Scheffe’s multiple-comparisons test was used after ANOVA.
AA and DHA are known to elicit opposing immunomodulatory actions (12, 13). Even though the ratio of AA to DHA in the formula, which is consistent with worldwide ranges of AA and DHA in breast milk, was favorable to AA (1.9:1), the final immunologic outcome (ie, suppressed immune responsiveness) was more consistent with the previously reported immunosuppressive effects of DHA (13), which suggested that DHA may have neutralized the ability of AA to enhance influenza virus–specific immune responses. AA/DHA-enriched formulas decreased antigen-specific responses against influenza virus on

### TABLE 6

<table>
<thead>
<tr>
<th>Nonvaccinated pigs</th>
<th>Vaccinated pigs</th>
<th>SEM</th>
<th>D</th>
<th>V</th>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>AA/DHA</td>
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<td>cpm stimulated wells/</td>
<td>cpm unstimulated wells</td>
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<td>Day 0</td>
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<tr>
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<tr>
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<tr>
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<td>340.42</td>
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<td>Day 23</td>
<td></td>
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<tr>
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<td>138.37a</td>
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<td></td>
</tr>
<tr>
<td>Virus</td>
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<td>691.24</td>
<td>239.71</td>
<td>251.22</td>
<td>164.23</td>
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1 D, the main effect of the diet; V, the main effect of vaccination, and D × V, the 2-factor interaction between the vaccination status and the dietary treatments; ConA, concavalin A. Least-squares mean values (n = 16, days 0–9; n = 8, days 10–30). Values in a row with different superscript letters are significantly different, P < 0.05.

2 On day 9, 8 blocks of 3 pigs each were vaccinated as described in Materials and Methods.

3 Before vaccination (eg, days 0 and 9), data were analyzed as a repeated-measures randomized complete-block design. After vaccination, data were analyzed as a repeated-measures factorial arrangement within a split-plot design (both analyses used ANOVA). Vaccination status represents the whole plot, and dietary treatments represent the subplot. The experimental unit for the whole plot was a block of 3 littermate pigs, and that for the subplot was 3 piglets within 1 block. The 3-factor interaction (vaccination × diet × time) was significant for the virus-specific proliferation (P < 0.001) but not for the mitogen (ConA)–induced proliferation. In addition, all three 2-factor interactions (D × V, P < 0.002; diet × time, P < 0.0006; and vaccine × time, P < 0.0002) were significant for the virus-specific proliferation but not for ConA–induced proliferation. Scheffe’s multiple-comparisons test was used after ANOVA.

AA and DHA are known to elicit opposing immunomodulatory actions (12, 13). Even though the ratio of AA to DHA in the formula, which is consistent with worldwide ranges of AA and DHA in breast milk, was favorable to AA (1.9:1), the final immunologic outcome (ie, suppressed immune responsiveness) was more consistent with the previously reported immunosuppressive effects of DHA (13), which suggested that DHA may have neutralized the ability of AA to enhance influenza virus–specific immune responses. AA/DHA-enriched formulas decreased antigen-specific responses against influenza virus on

![FIGURE 2. Least-squares mean (±SEM) antigen-specific proliferation of CD4+ T cells against influenza virus in cultures of peripheral blood mononuclear cells recovered from nonimmunized (A) and influenza virus–immunized (B) piglets fed control formula (□), arachidonic acid– and docosahexaenoic acid– (AA/DHA) enriched formula (****), or sow milk (○). Piglets were immunized with an inactivated influenza virus vaccine on day 9 of the experiment. n = 16 from day 0 to day 9; n = 8 from day 10 to day 30. The error bars in nonimmunized piglets or at early timepoints are smaller than the symbols and cannot be seen in the figure. Before the immunization treatment, data were analyzed as a repeated-measures randomized complete-block design. After the immunization treatment, data were analyzed as a repeated-measures factorial arrangement within a split-plot design. Immunization status represents the whole plot, and dietary treatments represent the subplot. The experimental unit for the whole plot was a block of 3 littermate pigs, and that for the subplot was 3 piglets within 1 block. The 3-factor interaction (diet × immunization × time) was not significant. However, the 2-factor immunization × time (P < 0.001) and immunization × diet (P < 0.04) interactions were significant. Scheffe’s multiple-comparisons test was used after ANOVA. *Significant differences among treatments attributed to the interaction between immunization and diet, P < 0.05.]
day 23, but it is important to note that AA/DHA-enriched formulas have shown no adverse effects on the immune response in infants (11, 34).

In a recent study from our laboratory that examined the nutritional interaction between CLA and n-3 PUFAs in experimental inflammatory bowel disease, we found that, when CLA was fed in combination with n-3 PUFAs, n-3 PUFAs abrogated the beneficial effects of CLA in clinical activity by blocking CLA-induced activation of peroxisome proliferator–activated receptor-γ (PPAR-γ; 35). In this regard, DHA has been found to suppress transactivation of PPAR-γ and expression of the PPAR-γ-responsive gene, CD36, by the synthetic PPAR-γ agonist ciglitazone in a colon tumor cell line (36). Thus, DHA may have blocked the ability of AA to modulate immune function through a similar molecular mechanism.

It is intriguing that the levels of lymphoproliferation in piglets fed AA/DHA-enriched formula did not differ significantly from those in sow-reared piglets. In this regard, it is known that the prevalences of atopies, eczema, and food allergies in infants and children have risen in parallel with the decrease in the practice of breastfeeding. We propose that AA and DHA may prevent or ameliorate autoimmune and allergic reactions in infants by down-modulating T cell responses. In support of this hypothesis, we found that IL-10 mRNA was upregulated on day 30 in piglets fed AA/DHA-enriched formula and sow-reared piglets. IL-10 is an antiinflammatory cytokine produced by induced regulatory T
cells, which has major suppressive effects on immune and inflammatory responses, and its absence or suppression in an area of the body constantly exposed to antigens (ie, intestine) leads to chronic inflammation (37). The upregulated IL-10 expression observed in piglets fed AA/DHA-enriched formula is consistent with the findings of a clinical study indicating that IL-10 production was lower in preterm infants fed control formula than in those fed an AA/DHA-enriched formula or human breast milk (11). However, because IL-10 expression was measured systemically in the current study, when the antigenic load is limited or null, the benefits of this effect are not clear, whereas potential disadvantages associated with suppressed systemic adaptive immune responses may include decreased resistance against bacterial or viral infection (or both). These findings suggest that future studies should aim at assessing the role of AA/DHA-enriched formula in preventing or ameliorating allergies, autoimmunity, and intestinal inflammation. Essentially, these data show that both the supplementation of the diet with AA and DHA and the feeding of sow milk during the neonatal period modulated antigen-specific T cell responses to an inactivated influenza virus and up-regulated IL-10 expression.

REFERENCES


ABSTRACT

**Background:** Zinc deficiency, cell-mediated immune dysfunction, susceptibility to infections, and increased oxidative stress have been observed in elderly subjects (ie, those >55 y old). Zinc is an effective antiinflammatory and antioxidant agent.

**Objectives:** The primary objective was to determine the effect of zinc on the incidence of total infections in healthy elderly subjects. The secondary objective was to determine the effect of zinc on cytokines and oxidative stress markers.

**Design:** A randomized, double-blind, placebo-controlled trial of zinc supplementation was conducted in elderly subjects. Fifty healthy subjects of both sexes aged 55–87 y and inclusive of all ethnic groups were recruited for this study from a senior center. The zinc-supplemented group received zinc gluconate (45 mg elemental Zn/d) orally for 12 mo. Incidence of infections during the supplementation period was documented. The generation of inflammatory cytokines, T helper 1 and T helper 2 cytokines, and oxidative stress markers and the plasma concentrations of zinc were measured at baseline and after supplementation.

**Results:** Compared with a group of younger adults, at baseline the older subjects had significantly lower plasma zinc, higher ex vivo generation of inflammatory cytokines and interleukin 10, and significantly lower in the zinc-supplemented than in the placebo group. Plasma zinc and phytohemagglutin-induced interleukin 2 mRNA in isolated mononuclear cells were significantly higher in the zinc-supplemented than in the placebo group.

**Conclusions:** After zinc supplementation, the incidence of infections was significantly lower, plasma zinc was significantly higher, and generation of tumor necrosis factor α and oxidative stress markers were significantly lower in the zinc-supplemented than in the placebo group.

**KEY WORDS** Elderly subjects, infections, interleukin 2 mRNA, zinc, oxidative stress, tumor necrosis factor α, interleukin 1β

INTRODUCTION

The essentiality of zinc and its deficiency in humans were recognized in the early 1960s (1, 2). Dietary zinc intake declines with advancing age in both developing and developed countries (3, 4). Zinc deficiency and susceptibility to infections due to cell-mediated immune dysfunction have been reported to occur in the elderly (3, 4).

Oxidative stress has been recognized as an important contributing factor in several chronic diseases attributed to aging, such as atherosclerosis and related cardiovascular disorders, mutagenesis and cancer, neurodegeneration, and immunologic disorders and even in the aging process itself (5). Together, O2−, H2O2, and ‘OH are known as reactive oxygen species, and they are continuously produced in vivo under aerobic conditions. Multiple roles of zinc as an antioxidant in cell cultures and animal models have been observed (5–8). The use of zinc in the management of oxidative stress in the elderly has not, however, been reported.

Inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin (IL) 1β, generated by activated monocytes and macrophages, are also known to generate greater amounts of reactive oxygen species (9, 10). In addition, chronic inflammatory processes have been implicated in high cardiovascular mortality in elderly subjects (11, 12). TNF neutralization in the treatment of septic shock (13, 14) and the use of IL-1 receptor antagonist (IL-1ra) and TNF antibody in the treatment of rheumatoid arthritis (15, 16) suggest that these cytokines are important in the pathogenesis of the above disorders. Increases in inflammatory cytokines TNF-α and IL-1β have been associated with cutaneous leishmaniasis (17), and increases in lipid peroxidation products were associated with zinc deficiency in children with chronic giardiasis (18).

Zinc supplementation to healthy human subjects aged 20–50 y reduced the concentrations of the oxidative stress–related by-products malondialdehyde (MDA), 4-hydroxynonenals (HAE), and 8-hydroxydeoxyguanine in the plasma; inhibited the ex vivo induction of TNF-α and IL-1β mRNA in mononuclear cells (MNCs); and provided protection against TNF-α–induced nuclear factor–κB activation in isolated MNCs (19). We previously provided evidence that, in the promyelocytic leukemia cell line...
HL-60, which differentiates to the monocyte and macrophage phenotype in response to phorbol-12-myristate-13-acetate, zinc increases the expression of A20 and the binding of A20 transactivating factor to DNA, which results in the inhibition of induced nuclear factor–κB activation (19). Nuclear factor–κB is involved in the gene expression of TNF-α and IL-1β in monocytes and macrophages in humans and HL-60 cells, and the effect of zinc in inhibiting the gene expression of TNF-α and IL-1β is cell specific (20–25).

Because zinc deficiency and susceptibility to infections due to cell-mediated immune dysfunctions have been observed in the elderly, we hypothesized that zinc supplementation would reduce the incidence of infection in the elderly. To understand the mechanism by which zinc may affect cell-mediated immune functions, we used reverse transcriptase (RT)–polymerase chain reaction (PCR) analysis to assess phytohemagglutinin-induced expression of IL-2 mRNA in isolated MNCs obtained from elderly subjects before and after supplementation. Because zinc supplementation to younger adults reduced the generation of inflammatory cytokines and decreased oxidative stress markers (19), we also hypothesized that zinc supplementation to the elderly not only would increase the generation of IL-2 mRNA in MNCs but also would decrease the generation of TNF-α and IL-1β and decrease oxidative stress markers.

SUBJECTS AND METHODS

Subjects

All subjects provided written informed consent. The study protocol was approved by the Human Investigation Committee of Wayne State University and was in accord with the Helsinki Declaration as revised in 1983.

Young adults

Thirty-one young adults (̅ ± SD age: 32.1 ± 13.1 y; range: 18–54 y) were recruited for assays of plasma zinc, intercellular adhesion molecule, vascular endothelial cellular adhesion molecules, E-selectin, nitric oxide (NO), MDA, and inflammatory cytokines generated ex vivo, so that their values could be compared with those of the older subjects in the current study. These studies were done only once.

Twenty-eight of these subjects were white, 2 were African American, and 1 was American Indian. Twelve were men and 19 were women. These subjects were affiliated with Wayne State University, and several were medical students. They were all free of any chronic illness, and none had any infection during the 6-mo observation period.

Elderly subjects

A diagram of the study protocol is shown in Figure 1. We recruited 50 healthy elderly adults of both sexes (aged 55–87 y) and all ethnic groups from St Patrick’s Senior Citizen Center (Detroit, MI) to participate in a randomized, placebo-controlled trial of the efficacy of zinc with respect to the incidence of infections and the effect on ex vivo–generated inflammatory cytokines and plasma concentrations of markers of oxidative stress. One participant in the zinc group dropped out on day 2. We therefore had complete data on 49 participants.

A complete chart review of each elderly subject was done by the research nurse to determine his or her potential eligibility for the study. Exclusion criteria were as follows: life expectancy (as estimated by the physician) of <8 mo; progressive neoplastic disease; severe cardiac dysfunction (New York Heart Association Class IV); significant kidney disease (blood urea nitrogen >40 mg/dL or creatinine >2.0 mg/dL); significant liver disease (known active hepatitis or cirrhosis) or transferrin concentrations >25% above the upper normal laboratory values; or serum alkaline phosphatase concentrations >200 IU. We excluded those persons who were self-supplementing with zinc, who were not mentally competent, or who did not understand the study information and could not provide informed consent.

The purpose of St Patrick’s Senior Citizen Center is to empower the elderly to live independently and with dignity and good quality of life. The center provides programs of health education and classes in art, computers, and a biweekly exercise program. Seniors are offered breakfast and lunch 7 d/wk. Transportation to and from their homes (if necessary) is provided daily and for weekly outings. Thus, the subjects from St Patrick’s were healthier than elderly persons living in nursing homes.

Intervention

Elderly subjects were randomly assigned in pairs to the zinc-supplemented or the placebo group with the use of envelopes that each contained 2 smaller envelopes (1 assigning a subject to zinc treatment and 1 assigning a subject to placebo). The persons caring for the patient, involved in drawing blood, or running laboratory analyses were blinded to the assignment. In the zinc group, 1 subject was 80 y old, 1 subject was 87 y old, 15 subjects
were <70 y old, and 7 were 70–80 y old. In the placebo group, 1 subject was 82 y old, 15 subjects were <70 y old, and 9 subjects were 70–80 y old.

Each day for 12 mo, subjects in the zinc-supplemented group received 1 capsule of zinc gluconate (15 mg elemental zinc) orally 1 h before breakfast and 2 capsules before going to bed (≥2 h after dinner or last meal). Subjects in the placebo group received placebo capsules in the same manner. Both zinc and placebo capsules were supplied by Labcatal Laboratories (Paris, France). The zinc content of capsules was checked and certified by Labcatal Laboratories.

Outcome measures

Our primary endpoint was to determine the incidence of infections in the 2 groups of subjects. To eliminate the effect of seasonal variations, participants were followed for a period of 12 mo. A nurse practitioner evaluated study subjects who appeared to have infections. Because a diagnosis of infection occasionally is difficult to establish in the elderly, given such subjects’ atypical presentation of disease, subtle clinical manifestations, and cognitive impairment, the practice guidelines for evaluation of fever and infections in long-term care facilities was used as our basis (26). The nurse-practitioner was blinded to the treatment assignment. All subjects were given an oral glass thermometer (Becton Dickinson, San Jose, CA) and a pocket calendar, and they were instructed to record infection symptoms and temperature on the calendar on a daily basis. All of the subjects had typical presentations, such as fever, sore throat, upper respiratory tract infection (URI), and common cold. Secondary endpoints were types of infection and laboratory variables such as plasma zinc, percentages of cells producing cytokines, and generation of cytokines and plasma oxidative stress markers.

Preliminary study

An additional 24 subjects were recruited from St Patrick’s Senior Citizens Center for a preliminary study. For 23 of these subjects, the ages ranged from 56 to 76 y, and 1 subject was 83 y old. Twelve were African American, and 12 were white. Eleven were men, and 13 were women. They were followed for 1 y for incidence of infection. With the use of the definition for infection as outlined above, the mean (±SD) incidence of infections was 1.4 ± 0.95/y in each of 24 zinc-nonsupplemented elderly subjects. We chose a sample size of 50 for the current study so that we could detect a 50% reduction in the incidence of infections in the zinc-supplemented group, with a SD of 0.95, a 2-sided P value of 0.05, and an approximate power of ≥80%.

Plasma zinc and copper

Plasma zinc was assayed by methods established in our laboratory that used flameless atomic absorption spectrophotometry with a Zeeman background corrector (SpectraAA 220Z; Varian Optical Spectroscopy Instruments, Victoria, Australia (19). Samples were digested in zinc-free nitric acid and diluted with zinc-free water before analysis. Reference standards included bovine liver (National Bureau of Standards) and pooled plasma previously analyzed by flame atomic absorption. The normal values for healthy persons are 110 ± 10 μg/dL, and values of <90 μg/dL (2 SDs below the mean in the current study) are considered to be in the deficient range. Plasma copper was also analyzed by flameless atomic absorption spectrophotometry.

Percentage of cells positive for specific cytokines

Whole blood (0.5 mL) was mixed with RPMI-1640 media supplemented with 10% fetal bovine serum and 10 μg brefeldin-A/mL and then incubated at 37 °C for 4 h in the presence of selected stimulators. For T helper 1 (Th1) cytokines [IL-2 and interferon (IFN) γ], we used phorbol 12-myristate-13-acetate (25 ng/mL) and ionomycin (1 μg/mL); for T helper 2 (Th2) cytokines (IL-4 and IL-10), we used 25 μg concanavalin/mL; and for inflammatory cytokines (IL-1β and TNF-α), we used 2 μg lipopolysaccharide/mL (LPS; Sigma Aldrich, St Louis, MO). The source of the LPS was Escherichia coli 0111:B4. After incubation, samples were labeled with fluorescence-labeled anti-CD3 (for Th1 and Th2 cytokines) or anti-CD14 (for IL-1β and TNF-α), lysed, and then fixed with 0.25 mL of 3.7% formaldehyde in phosphate-buffered saline. Fixed cells were permeabilized with 0.1% saponin (Sigma Chemical Co, St Louis, MO) and labeled with antibodies directed against specific cytokines. All fluorescence-labeled antibodies were obtained from CalTag Laboratories (Burlingame, CA). Data for samples were collected at the flow cytometry core facility at the Wayne State University Karmanos Cancer Institute, and the results were analyzed by using CELL QUEST software (version 3.2; Becton Dickinson).

Ex vivo generation of cytokines

MNCs were isolated from 10 mL heparinized whole blood by using Histopaque 1077 density gradient (Sigma Chemical Co) and then resuspended in RPMI-1640 medium plus 10% fetal bovine serum (1 × 10⁶/mL). Cells were stimulated with phytohemagglutinin for 48 h for the generation of Th1 and Th2 cytokines and with LPS for 24 h for the generation of inflammatory cytokines (25). Supernatants were removed and stored at −20 °C until they were assayed with the use of an enzyme-linked immunosorbent assay for IL-1β, IL-2, IL-4, IL-10, IFN-γ, and TNF-α (R&D Systems, Minneapolis, MN).

Markers of oxidative stress

Plasma lipid peroxidation by-products MDA and HAE were measured by using a thiobarbituric acid lipid peroxidation assay kit (Oxford Biochemical Research, Oxford, MI); plasma 8-hydroxydeoxyguanaine was assessed with the use of an enzyme-linked immunosorbent assay (Japan Institute for the Control of Aging, Shizuoka, Japan), and total plasma NO was assessed by using an NO kit (Oxford Biomedical) at baseline and after 6 mo of supplementation.

Reverse transcriptase–polymerase chain reaction for interleukin 2 mRNA

We selected 12 zinc-deficient subjects for the study of reverse transcriptase–PCR for IL-2 mRNA. They were a separate group of subjects and not a part of the main study. The inclusion and exclusion criteria were the same as those described in detail for elderly subjects. These subjects were recruited from the senior center; they provided written informed consent and were randomly assigned to receive either zinc or placebo according to the protocol. The mean age of the 6 subjects in the zinc group was 65 ± 9.1 y (range: 56–83 y), and that of the 6 subjects in the placebo group was 67.5 ± 6.6 y (range: 61–81 y; P = 0.6). The zinc group included 4 women and 2 men; the placebo group included 3 women and 3 men. The zinc group included 4 whites, 1 African American, and 1 Chinese; the placebo group included
4 whites and 2 African Americans. The mean plasma zinc concentrations at baseline in the zinc and placebo groups were 86.38 ± 3.6 and 88 ± 0.82 μg/dL, respectively (P = 0.35).

We compared the baseline data for IL-2 mRNA and plasma zinc concentrations in these 12 zinc-deficient subjects with the data in a separate group of 12 zinc-sufficient subjects who were not a part of the main study. These subjects were also recruited from the senior center, were ambulatory, and were free of any chronic illness; they provided written informed consent. Their mean age was 65 ± 5.8 y (range: 59–83 y). This group included 3 men and 9 women and 8 whites, 3 African Americans, and 1 American Indian. Their mean plasma zinc concentration at baseline was 94.5 ± 2.54 μg/dL, whereas that in the 12 zinc-deficient subjects was 85.4 ± 2.54 μg/dL (P = 0.0001). At the baseline, the IL-2 mRNA of the zinc-sufficient subjects was 0.59 ± 0.05 and that of the zinc-deficient subjects was 0.39 ± 0.06 (P = 0.0001).

Isolated MNCs from each subject were stimulated with 10 μg/ml PHA-p for 24 h to induce IL-2 mRNA. Total RNA was extracted by using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. cDNA was prepared from 2 μg total RNA by RT at 42 °C for 30 min in a 20-μL reaction and synthesized in the presence of Moloney murine leukemia virus RT (2.5U) by using 2.5 μl mol oligo-d (thymidine) primer/L and reaction conditions as described by the manufacturer (Invitrogen). The reaction was stopped by heating the samples to 99 °C for 5 min. A GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA) was used for cDNA amplification. For the IL-2 cDNA PCR reaction, cDNA was amplified in a total of 50 μL in the presence of 25 pmol/L of each oligo nucleotide 5’ TGT ACA GGA TGC AAC TCC TG and 3’ CAA TGG TTG CTG TC TCA TCA G. Reaction conditions were melting for 45 s at 94 °C, annealing for 45 s at 60 °C, and extension for 120 s at 72 °C for 35 cycles, which were followed by a final extension for 7 min at 72 °C in a thermal cycler (Perkin-Elmer, Wellesley, MA).

### Statistical analysis

Demographic differences between the zinc and placebo groups were examined by t test (age) and chi-square test (sex and ethnicity). For other variables, t tests were used to compare group differences when variables were normally distributed. If distributions were not normal, the group differences were compared by using the nonparametric Wilcoxon rank-sum test. Chi-square tests (Fisher’s exact test: 2 × 2 frequency table) were used to compare the incidence of infections in the zinc and placebo groups. In both the zinc and placebo groups, the changes in laboratory variables from baseline to after intervention were compared by using a paired t test (27). Multivariate repeated-measures analyses were used to examine measures over time. All statistical analyses were conducted with JMP software (version 5.0; SAS Institute Inc, Cary, NC) on a Macintosh Powerbook G4 computer (Apple Computers, Cupertino, CA).

### RESULTS

The demographic characteristics of the elderly participants in the zinc-supplemented and placebo-supplemented groups are shown in Table 1. Neither the ages of the 2 groups or the number of men and women participating in each group differed significantly. An equal number of African Americans and whites participated in the 2 groups. No other variables differed significantly between the groups.

<table>
<thead>
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<tr>
<td>Procardia</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>Celebrex</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Proventil and Brethine</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>Anticonvulsant</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Lipitor</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Antidepressant (Zoloft)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Synthroid</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>Influenza vaccine (n)</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>Pneumonia vaccine (n)</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Chronic disease (n)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Hypothyroid</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Hypertension</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>Smoker ≥1 pack/d</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Alcohol (regular use)</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

### Table 1

Demographic characterization of elderly study participants

A comparison of baseline data between the younger subjects and the elderly subjects is shown in Table 2. Plasma zinc was lower and the percentage of cells producing IL-1β and TNF-α and the generated concentrations of these cytokines were significantly higher in the elderly subjects. Intercellular adhesion molecules, vascular endothelial cell adhesion molecules, and E-selectin in the plasma also were significantly higher in the elderly. IL-10 generated by Th2 cells, which are known to produce a negative effect on IL-2 generated by Th1 cells) was significantly higher in the elderly. The oxidative stress markers also were significantly higher in the elderly than in the younger adults.

The effect of supplementation on clinical variables is shown in Table 3. The mean incidence of infections per subject in 12 mo was significantly (P < 0.01) lower in the zinc-supplemented group (0.29 ± 0.46) than in the placebo group (1.4 ± 0.95; effect size: 1.46). When the infections were categorized separately as URI (which included rhinitis, sinusitis, and bronchitis), tonsillitis, common cold, cold sores, eye infection and flu, a significantly lower incidence of fever and a nonsignificant trend toward a lower incidence of the common cold were observed in the zinc-supplemented group than in the placebo group (28). The diagnosis of common cold was based on cough, sore throat, hoarseness, rhinorrhea, sinus tenderness, tachypnea, or rales. Seventeen subjects in the zinc-supplemented group had no evidence of any
EFFECT OF ZINC SUPPLEMENTATION IN THE ELDERLY

TABLE 2
A comparison of selected variables in young adults (18–54 y old) and in older subjects (>55 y old)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Young adults</th>
<th>Older subjects</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma zinc (µg/dL)</td>
<td>101.4 ± 10.0 (31)¹</td>
<td>94.3 ± 11.4 (49)</td>
<td>0.046</td>
</tr>
<tr>
<td>Plasma ICAM-1 (ng/mL)</td>
<td>538 ± 112.7 (25)</td>
<td>652.6 ± 169.8 (47)</td>
<td>0.001</td>
</tr>
<tr>
<td>Plasma VCAM-1 (ng/mL)</td>
<td>1766 ± 480.4 (25)</td>
<td>2209 ± 890.5 (46)</td>
<td>0.008</td>
</tr>
<tr>
<td>Plasma E-selectin (ng/mL)</td>
<td>32.2 ± 13.1 (19)</td>
<td>84.6 ± 47.6 (69)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma NO (µmol/L)</td>
<td>42.7 ± 10.9 (24)</td>
<td>55.6 ± 14.7 (36)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma MDA (µmol/L)</td>
<td>0.36 ± 0.10 (16)</td>
<td>0.49 ± 0.15 (34)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

¹ ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular endothelial cellular adhesion molecule 1; NO, nitric oxide; MDA, malondialdehyde; IL, interleukin; TNF-α, tumor necrosis factor α.

TABLE 3
Effect of zinc and placebo supplementation on clinical variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>Zinc group (n = 24)</th>
<th>Placebo group (n = 25)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infection</td>
<td>29</td>
<td>88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Upper respiratory tract infection</td>
<td>12</td>
<td>24</td>
<td>0.136</td>
</tr>
<tr>
<td>Tonsillitis</td>
<td>0</td>
<td>8</td>
<td>0.255</td>
</tr>
<tr>
<td>Common cold</td>
<td>16</td>
<td>40</td>
<td>0.067</td>
</tr>
<tr>
<td>Cold sores</td>
<td>0</td>
<td>12</td>
<td>0.124</td>
</tr>
<tr>
<td>Flu</td>
<td>0</td>
<td>12</td>
<td>0.124</td>
</tr>
<tr>
<td>Fever</td>
<td>0</td>
<td>20</td>
<td>0.027</td>
</tr>
<tr>
<td>One infection each/y</td>
<td>29</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Two infections each/y</td>
<td>0</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Three infections each/y</td>
<td>0</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Four infections each/y</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Received antibiotics</td>
<td>8</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

¹ Each subject could appear in >1 subcategory of infections.

In the zinc-supplemented group, the total incidence of infections was 35 (8 from January through March, 11 from April through December). In the placebo group, the total incidence of infections was 35 (8 from January through March, 11 from April through December). In the zinc-supplemented group, the percentage of cells positive for TNF-α, IL-1β, or IL-10 did not change significantly with time in either the placebo or zinc-supplemented group.

The changes in concentrations of plasma molecular markers of oxidative stress (MDA + HAE and 8-oHdG) between baseline and at the end of 6 mo of zinc supplementation showed a greater (and significant) decrease in the zinc-supplemented group than in the placebo group (Table 4). A nonsignificantly lower concentration of NO was observed in the zinc group at the end of 6 mo. We did not repeat these studies at the end of 12 mo of supplementation.

In MNCs isolated from zinc-deficient elderly subjects, zinc supplementation increased the ex vivo PHA-induced IL-2 mRNA expression and plasma zinc concentration above the values found in the zinc-deficient subjects who were given placebo (P < 0.05; Table 7). At baseline, the plasma zinc and IL-2 mRNA concentrations did not differ significantly between the zinc and placebo group subjects (Table 7).

Group × time interactions were significant for IL-2 mRNA (P = <0.0001) and plasma zinc (P = <0.0088) concentrations.

TABLE 4
Effect of zinc (n = 24) and placebo (n = 25) supplementation on plasma zinc and copper concentrations

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>At 12 mo</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc group (µg/dL)</td>
<td>92.9 ± 9.45¹</td>
<td>104 ± 16.69</td>
<td>0.0002</td>
</tr>
<tr>
<td>Placebo group</td>
<td>95.7 ± 13.09</td>
<td>88.5 ± 9.66</td>
<td></td>
</tr>
<tr>
<td>Plasma copper (µg/dL)</td>
<td>182.2 ± 50.5²</td>
<td>210.7 ± 60.7</td>
<td>0.750</td>
</tr>
<tr>
<td>Placebo group</td>
<td>193.4 ± 61.6</td>
<td>215.4 ± 58.7</td>
<td></td>
</tr>
</tbody>
</table>

¹ No significant differences in plasma zinc or plasma copper were found between the 2 groups at baseline (t test).

² P value for change in groups over time (time × group interaction) (multivariate repeated-measures analyses).

² ± SD (all such values).
In the zinc-supplemented group, both of these variables increased during the 6-mo period, whereas they did not change in the placebo-treated group (Table 7). During the 6-mo observation period, 2 subjects in the zinc-treated group had one episode of bronchitis each. In the placebo-treated group, 4 subjects had an infection: 1 subject each had laryngitis, flu, URI, and common cold.

**DISCUSSION**

Our study showed that zinc supplementation administered to the elderly population resulted in a significant decrease in the incidence of infection. Many animal studies show that zinc deficiency decreases resistance to a range of bacterial, viral, fungal, and parasitic pathogens (29), probably because of the immune impairment induced by zinc deficiency. The baseline plasma
zinc concentrations in our elderly subjects were low, which suggested that a marginal zinc deficiency was present in this group. Thus, the enhancing effect on the immune response of providing zinc should translate into improved host defense and increased resistance to pathogens in zinc-deficient subjects. According to our plasma zinc criteria, ≈35% of the elderly subjects in the current study were considered zinc-deficient. We observed, however, that zinc supplementation also decreased concentrations of oxidative stress markers and showed antiinflammatory responses in subjects who were not zinc deficient as judged by plasma zinc concentrations at baseline, which suggested that zinc had a therapeutic effect in this regard similar to the effect we had earlier reported in healthy volunteers (19).

Zinc deficiency not only adversely affects the production of IL-2 and IFN-γ but also decreases the IL-12 production from macrophages (AS Prasad, personal observations, 2002). IFN-γ along with IL-12 is required for optimal phagocytic activity of immune cells, including neutrophils, monocytes, macrophages, natural killer cells, and B and T cells. Zinc-deficient subjects have a greater susceptibility to a variety of pathogens (36). Results from our previous research indicate that nearly 30–35% of the upper middle-class elderly population in the Detroit area may be zinc deficient, as judged by their lymphocyte zinc concentrations (37). Low thymulin activity and low IL-2 production have been reported in zinc-deficient elderly subjects (36). Other investigators have observed that zinc supplementation to elderly subjects increased the numbers of circulating T cells in association with improved delayed-type hypersensitivity reactions and immunoglobulin G antibody responses to tetanus toxoid (38).

We have presented evidence for a significant increase in IL-2 mRNA in zinc-deficient elderly subjects after zinc supplementation, a finding consistent with our previous reports in experimental human model studies and in cell culture studies in the HUT-78 cell line (19, 39). We conclude that zinc is involved in increasing IL-2 production in zinc-deficient elderly subjects by increasing the gene expression of IL-2. We have also observed a decrease in IL-10 production in zinc-supplemented elderly subjects, and this decrease may also have an effect of increasing IL-2 production (40).

Results from the current study show that elderly subjects are oxidatively stressed and that zinc is an effective antiinflammatory as well as an antioxidant agent. The administration of 45 mg elemental zinc/d did not reduce plasma copper concentrations in zinc-supplemented elderly subjects. Inasmuch as zinc is nonmutagenic and relatively nontoxic (except for induction of copper deficiency when the therapeutic dose of zinc is >50 mg elemental zinc/d and is used for >12 wk), a long-term trial of zinc in the elderly should be undertaken to determine zinc’s role in preventing many chronic disorders that have been related to oxidative stress and chronic inflammatory cytokines such as TNF-α, IL-1β, and IL-8 (13–16). Most important, we show here that the ex vivo generation of TNF-α from isolated MNCs is significantly decreased in elderly subjects after zinc supplementation. We also observed in the placebo group that TNF-α increased with time, which suggests that this increase may be an effect of increasing age. A long-term, still ongoing trial has been conducted for the past 10 y in patients with age-related macular degeneration who received 80 mg elemental zinc as oxide and 2 mg Cu to prevent copper deficiency (41). Zinc alone has decreased the incidence of blindness due to age-related macular degeneration in 25% of these elderly subjects, and, most important, increased longevity has also been reported in those treated with zinc (41, 42). These observations are truly exciting and deserve further exploration.

The limitation of the current study is that the number of subjects in the trial was small. We hope that our results will stimulate larger zinc trials in the elderly.

The authors thank Nimisha Doshi for technical assistance.

ASP was responsible for the conception and design of the study and for obtaining funding; DS, FWJB, BB, JDS, and LJC were responsible for recruitment of subjects and provision of the study materials; JTF, FWJB, and BB were responsible for collecting and organizing the data; ASP, JTF, FWJB, and BB were responsible for the analysis and interpretation of data; JT Fitzgerald was responsible for the statistical analysis; ASP and JTF drafted the manuscript; ASP, FWJB, and BB were responsible for revising the manuscript; and ASP was responsible for final approval of the manuscript. None of the authors had any personal or financial conflict of interest.

REFERENCES

21. Prasad AS, Bao B, Beck FWJ, Sarkar FH. Zinc activates NF-
22. Krikos A, Laherty CD, Dixit VM. Transcriptional activation of the tumor
14. Pennington JE. Therapy with antibody to tumor necrosis factor in sepsis.
11. Ward PA. Cytokines, inflammation, and autoimmune diseases. Hosp
8. Bettger WJ. Zinc and selenium, site-specific versus general antioxidant.
5. Castro L, Freeman BA. Reactive oxygen species in human health and
20. Prasad AS, Bao B, Beck FWJ, Sarkar FH. Zinc enhances the expression
18. Demirci M, Delibas N, Altuntas I, Oktem F, Yonden Z. Serum iron, zinc
16. Opal SM, DePalo VA. Impact of basic research on tomorrow's medicine:
and monocyte redox-sensitive genes by AG1–1067: a novel antioxidant and
11. Ozaki Y, Ohashi T, Kume S. Potentiation of neutrophil function by
9. Ozaki Y, Ohashi T, Kume S. Potentiation of neutrophil function by
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25. Song HY, Rothe M, Goeddel DV. The tumor necrosis factor-inducible
23. Clemons TE, Kurinij N, Sperduto RD; AREDS Research Group. Asso-
ciations of mortality with ocular disorders and an intervention of high-
22. Krikos A, Laherty CD, Dixit VM. Transcriptional activation of the tumor
21. Prasad AS, Bao B, Beck FWJ, Sarkar FH. Zinc activates NF-
20. Prasad AS, Bao B, Beck FWJ, Sarkar FH. Zinc enhances the expression
18. Demirci M, Delibas N, Altuntas I, Oktem F, Yonden Z. Serum iron, zinc
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16. Opal SM, DePalo VA. Impact of basic research on tomorrow’s medicine:
comparison of chimeric monoclonal antibody to tumor necrosis factor
226. Heyninck K, Beyaert R. The cytokine-inducible zinc finger protein A20
14. Pennington JE. Therapy with antibody to tumor necrosis factor in sepsis.
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11. Ozaki Y, Ohashi T, Kume S. Potentiation of neutrophil function by
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226. Heyninck K, Beyaert R. The cytokine-inducible zinc finger protein A20
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9. Ozaki Y, Ohashi T, Kume S. Potentiation of neutrophil function by
8. Bettger WJ. Zinc and selenium, site-specific versus general antioxidant.
5. Castro L, Freeman BA. Reactive oxygen species in human health and
Low birth weight is associated with altered immune function in rural Bangladeshi children: a birth cohort study1–3

Rubhana Raqib, Dewan S Alam, Protim Sarker, Shaikh Meshbahuddin Ahmad, Gul Ara, Mohammed Yunus, Sophie E Moore, and George Fuchs

ABSTRACT

Background: Low birth weight is generally an outcome of a fetal insult or nutritional insufficiency. Recent studies have shown that such exposure early in life may have long-term implications for later immunocompetence and susceptibility to infectious diseases.

Objective: We aimed to investigate the effect of birth weight on immune function in preschool-age children.

Design: A birth cohort cross-sectional study was conducted in children (n = 132) aged 60.8 ± 0.32 mo who were born in Matlab, a rural area of Bangladesh, and whose weight and length were measured within 72 h of birth. The outcome measures were thymopoiesis, T cell turnover, acute phase response, and percentage of lymphocytes.

Results: Children born with low birth weight (<2500 g; LBW group, n = 66) had significantly higher concentrations of T cell receptor excision circles in peripheral blood mononuclear cells—a biomarker for thymopoiesis—and significantly higher serum bactericidal activity and C-reactive protein concentrations than did children born with normal birth weight (≥2500 g; NBW group, n = 66) (P < 0.05 for both). The LBW group children had significantly lower concentrations of interleukin 7 in plasma (P = 0.02), shorter telomere length in peripheral blood mononuclear cells (P = 0.02), and a lower percentage of CD3 T cells (P = 0.06) than did the NBW group children.

Conclusions: Greater peripheral T cell turnover (shorter telomeres and lower CD3 concentrations) due to immune activation (elevated C-reactive protein concentrations and bactericidal activity) may have resulted in a greater need for replenishment from the thymus (higher T cell receptor excision circles); these events may cause lower immune functional reserve in preschool-age children born with LBW. Thus, LBW has implications for immunocompetence and increased vulnerability to infectious diseases in later life. Am J Clin Nutr 2007;85:845–52.

KEY WORDS Low birth weight, T cell receptor excision circles, TRECS, telomere, CD3 T cells, C-reactive protein

INTRODUCTION

Low birth weight (LBW) can be the product of prematurity or of intrauterine growth retardation (IUGR). Whereas IUGR has several different causes, it often is the result of poor nutrition during pregnancy (1). Morbidity and mortality of LBW infants due to infectious diseases are known to be high (1), which has led researchers to explore the effect of LBW on immune function. Although many of the early studies focused on the depressed immune responses of LBW infants (2–7), more recent research suggests that these defects may persist beyond infancy (8, 9).

In a series of studies, Barker et al (10) introduced the hypothesis of the developmental origin of health and disease. This hypothesis postulates that conditions that most probably are nutritional program the fetus for development of chronic noncommunicable diseases in adulthood. In parallel, evidence is accumulating to suggest that early events may also program immune function. Studies in The Gambia in West Africa have shown associations between birth during the nutritionally poor “hungry” season and a greater risk of premature adult mortality due to infectious diseases (9). Further prospective studies in that population showed seasonal effects on thymic size (11) and function (12). A longitudinal study of adolescents in the Philippines provided evidence that prenatal undernutrition is significantly associated with low thymopoietin production and less growth in length during the first year of life and is positively associated with adolescent thymopoietin production (13). These findings add to a growing body of evidence that events occurring in utero or early in life might permanently affect components of the immune system, especially the thymus.

The aim of the current study was to explore the hypothesis that LBW may impair long-term immune function and that—in a tropical setting with high infectious exposure that will lead to persistent activation of the immune system, increased peripheral infiltration of thymus-derived naive T cells, and premature lymphocyte senescence—the effect may not become apparent until the later years of life. To test this hypothesis, we have assessed thymic output, T cell turnover, and lymphocyte proliferation...
response in peripheral blood lymphocytes of children from rural Bangladesh born at either LBW or a normal birth weight (NBW).

SUBJECTS AND METHODS

Study design

The study was conducted at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) field research station in Matlab, a typical rural and riverine delta area of Bangladesh, located ≈ 45 km from the capital, Dhaka. The current study selected participants from a cohort of infants born in Matlab during 1995 or 1996, full details on whom can be found elsewhere (14, 15). Birth weight was measured within 72 h of delivery by using a pediatric balance-beam scale (Seca, Hamburg, Germany) that is accurate to 10 g. The infant’s recumbent length was measured by using a regularly validated, constructed length board accurate to the nearest 0.1 cm. Infants’ weight and length measurements were compared with those of the National Center for Health Statistics reference population and converted to age- and sex-standardized z scores with the use of ANTHRO software [version 1.02 (Y2K-compliant); developed jointly by the World Health Organization, Geneva, Switzerland, and the Centers for Disease Control and Prevention, Atlanta, GA).

Study subjects

The current study aimed to compare children born as singletons and at full term with NBW (≥2500 g) with those born at LBW (<2500 g). Children who had known chronic illness or recent episode of measles were excluded from the study. The availability of the children for the current study was initially confirmed from the Matlab Health and Demographic Surveillance System records. A door-to-door survey was conducted to confirm the availability of the children; then, from the list of available children (whose families had not migrated), the required number of children was selected for the study. Initial screening in Matlab villages found 358 children who were available. For the study, sample size was calculated based on 5% type I error and 20% statistical power, with consideration of several outcome variables, such as total lymphocyte count, total leukocyte count, percentage of CD4 and CD8 T cells, the ratio of CD4 to CD8 cells, and the concentration of total immunoglobulin G (IgG). Because comparison of the immune variables—eg, T cell receptor excision circles (TRECs), telomeres, and C-reactive protein (CRP)—that are important for functional outcomes between LBW and NBW children has not previously been reported, it was of interest to explore these variables. A difference of 5% in the outcome variables between the 2 groups was considered to be important. We calculated that 66 subjects/group would be sufficient for the proposed study. From the cohort of 358 children, systematic random sampling was done in which every 2nd child was selected (the first child was chosen randomly), and a total of 179 children were selected (Figure 1). Thereafter, children were stratified into 2 groups: one consisting of children born with LBW (LBW group, n = 75) and the other with NBW (NBW group, n = 104). From each of these 2 groups, 66 children were randomly selected. Stratification into 2 groups, blinding, and decoding of the subjects was done by a person without relation to the study.

Anthropometric measurements at follow-up [weight, height, and midupper arm circumference (MUAC)] were taken by trained field research assistants. Weight was measured with a portable electronic scale (SECA) to the nearest 0.1 g. Height was measured to the nearest 0.1 cm with a locally made, wooden height stick. Any history of diarrhea or acute respiratory infection (upper and lower respiratory tract infections including asthma) and skin infections within the past 2 wk or current infection were also recorded. All instruments were validated by the supervisor of the field research assistants before use. Physical examination was conducted, including body temperature, respiratory rate, heart rate, pulse, and blood pressure measurements. For anthropometric measurements at follow-up, standard guidelines were followed by using growth percentile curves from the National Center for Health Statistics.

Written informed consent was obtained from the guardian of each child. The study was approved by the ethics review committee of the ICDDR,B: Center for Health and Population Research.

Sample collection

Fasting peripheral venous blood samples were obtained from each child at enrollment in the Matlab hospital laboratory. Blood samples were collected in heparin-coated sterile vials (Vacutainer; Becton Dickinson, Rutherford, NJ) and were processed to isolate peripheral blood mononuclear cells (PBMCs) from blood on Ficoll-Paque (Pharmacia-Upjohn, Uppsala, Sweden) by density gradient centrifugation at 1800 rpm at 24 °C for 25 min (Sorvall Legend RT; Kendro Laboratory Products GmbH, Hanau, Germany). A portion of the PBMCs was used for the lymphocyte proliferation assay, and a portion was stored in liquid nitrogen until used for flow cytometry and DNA extraction. Plasma collected as supernatant from the gradient was stored at −70 °C until it was used. Serum samples were also collected, and they were stored at −70 °C.

C-reactive protein and interleukin 7

Plasma concentrations of the hepatically derived acute phase protein CRP were used to detect ongoing infection or inflammation. CRP concentrations were measured by immunoturbidimetric assay with the use of a Hitachi 902 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Concentrations of
interleukin 7 (IL-7) in plasma were measured with a high-sensitivity colorimetric enzyme-linked immunosorbent assay (IL-7 immunoassay kit; R&D Systems QuantikineHS, Minneapolis, MN) according to the manufacturer’s instructions. The lower limit of detection was 0.1 pg/mL. Plasma IL-7 concentrations were measured to assess the effect of the cytokine IL-7 on thymopoiesis or the T cell regeneration process.

**Serum bactericidal response**

Preterm and small-for-gestational-age (SGA) infants have low amounts of neutralizing antibodies (7). Because enteric diarrheal diseases are common in rural Bangladesh, the serum bactericidal response was evaluated against a nonpathogenic *Escherichia coli* K12 strain, which lacks the O surface antigens (16). The serum bactericidal assay similar to that used for *Shigella* was modified to optimize it for nonvirulent *E. coli* K12 (17). Bacteria grown in Mueller Hinton broth (MHB; Difco, Detroit, MI) at 37 °C for 3 h were suspended in saline to an absorption of 0.4 at 600 nm [1 × 10⁸ colony-forming units (CFU)/mL], which was followed by serial dilution to a concentration of 1 × 10⁵ CFU/mL. Guinea pig complement (1:5) was added to the bacterial suspension, and the mixture was applied to microtiter plates (Nunc, Roskilde, Denmark) containing serum samples, with a starting dilution of 1:10, that were complement-inactivated by heat treatment and serially diluted by 50%. The volume of 200 μL/well was adjusted with MHB, and the plate was incubated in a shaker incubator (200 rpm) at 37 °C for 16 h. The optical density of the plates was measured at 595 nm. The titer of the serum was defined as the reciprocal of the last dilution in which no growth was evident by visual inspection (17).

**Lymphocyte proliferation response**

The ability of lymphocytes to proliferate polyclonally on activation with mitogens provides a tool for the assessment of lymphocyte function. The assay was conducted in triplicate on microtiter plates as described previously (18). PBMC suspensions (3 × 10⁸ cells/well) were incubated for 3 d in 37 °C and 5% CO₂ with the mitogen phytohemagglutinin (PHA (at 5 mg/L); Sigma, St Louis, MO) or without stimulant (RPMI 1640; Gibco). After 37 °C and 5% CO₂ with the mitogen phytohemagglutinin (PHA (at 5 mg/L); Sigma, St Louis, MO) or without stimulant (RPMI 1640; Gibco). After being washed in phosphate-buffered saline (PBS; pH 7.4), cells were suspended (1 × 10⁵ cells/mL) in PBS containing 2% heat-inactivated pooled human AB serum and were stained with triple combinations of monoclonal antibodies for 30 min at 4 °C. Cells were washed and resuspended in PBS containing 1% paraformaldehyde and analyzed within 4 h. Negative controls were included in each experiment. Three-color fluorescent analysis was performed by using a fluorescent cell sorter (FACSCalibur; Becton Dickinson, San Jose, CA). Fluorescein isothiocyanate– (FITC) labeled IgG2b and phycoerythrin-labeled IgG1 were used as isotype control. Before data acquisition, instrument settings were checked and optimized by using CalibRITE beads (Becton Dickinson). Data acquisition and analysis were done with CELL QUEST software (version 3.3; Becton Dickinson). All samples were analyzed by setting appropriate forward- and side-scatter gates around the lymphocytes, and the percentage of positive cells was estimated.

**Monoclonal antibodies**

The following antibodies were used in the study for the phenotype analysis by 3-color flow cytometry: anti–CD45 (pan leukocyte), anti–CD14 (monocytes), IgG1, IgG2 (negative isotype control), anti–CD3 [pan T cells; FITC, peridinin chlorophyll protein (PerCP), and antigen-presenting cells (APC)], anti–CD4 (helper/inducer T cells and FITC), anti–CD8 (suppressor/cytotoxic T cells, phycoerythrin, and PerCP), anti–CD69 (early activation marker and PerCP), anti–CD45RA (naïve T cells and FITC), anti–CD45RO (memory T cells and phycoerythrin), anti–CD56 (neural cell adhesion molecules, pan-natural killer cells, and phycoerythrin), anti–CD16 (natural killer cells, Fcy receptor III, and FITC). All antibodies were purchased from Becton Dickinson.

**Quantification of signal-joint T cell receptor excision circles by real-time polymerase chain reaction**

The thymus, a glandular organ, is the site of T cell differentiation and regeneration. Thymic T cell production can be assessed by measuring TREC as a traceable molecular marker in newly produced naïve T cells (19). Thus, the content of TREC in peripheral T cells is an indicator of thymopoiesis or newly synthesized and exported naïve T cells (19).

From the frozen whole-blood samples, DNA was isolated by using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. Quantification of signal-joint (sj) TREC was performed by using SYBR Green real-time quantitative PCR and an iCycler thermocycler (Bio-Rad version – 3.021; Life Science Research Group, Hercules, CA). The following primers were used: [forward primer > 5’ AAGAGGGCGACGCCCTCTCCAAGGCAAA 3’ and reverse primer < 5’ AAGCTGTAGTTGCAGCATTTGTGCCTCCG 3’]. Using natural 96-well plates that can be separated into 24- or 48-wells sections (semi-skirt; Nunc), a master mix was prepared that consisted of 12.5 μL Quantitect-SYBR Green (Qiagen), 0.5 μL forward and reverse primers each, 1.0 μL MgCl₂ (50 mmol/L), 1.0 μL of each primer pair, and 8.5 μL deionized water. Then, 23 μL of this master mix was added to the 96-well plate, which was followed by 2 μL of standards, samples, and...
negative controls in corresponding tubes to obtain a 25-μL reaction volume. Real-time polymerase chain reaction (PCR) was then performed under the following conditions: denaturation (1 cycle) at 95 °C for 3 min; preamplification of 40–45 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 45 s; and a second amplification (final extension step) cycle at 72 °C for 5 min. A standard (donated by PT Ngom) was prepared by using serial dilutions of a known number of copies of a fragment of the sjTREC gene sequence and included in each iCycler run to generate a standard curve (12). Serial dilutions of the cloned TREC-PCR product were used as a standard for absolute quantification of TREC concentrations. Samples were analyzed in duplicate. The number of copies of sjTRECs in the samples was automatically measured by reading off the standard curves generated and expressed as TREC content (ie, no. of copies) per 10⁶ PBMCs.

Telomere length

Telomeres are specialized structures at the ends of chromosomes. The length of the telomeres was reduced progressively during each cell replication cycle, and, thus, telomere length was shown to reflect the replicative history of those cells (20). After extraction of DNA as above, DNA was rehydrated overnight at room temperature, quantified by absorbance spectrophotometry at 260-nm wave length, and stored at 4 °C. The mean length of the telomeric restriction fragment (TRF) was determined by using the TeloTAGGG Telomere Length Assay (Roche, Basel, Switzerland) and by Southern blot. After exposure of the blot to an X-ray film, the TRFs were visualized. For quantitative measurements of mean TRF length, the signal intensity was scanned and read by an imaging system with the use of QUANTITY ONE software (version 4.2.2; BioRad Laboratories). The mean TRF length was estimated as the midpoint of the smear on each lane relative to a molecular-weight standard that was run in each gel. Results are given as mean TRF length of the sum equation of the telomere signal distribution.

Statistical analysis

Statistical analyses were done by using SIGMASTAT statistical software (version 3.1; Jandel Scientific, San Rafael, CA) and SPSS for WINDOWS software (Release 10; SPSS Institute, Chicago, IL). Data were expressed as means ± SEs. Continuous variables were compared between groups by using the Student’s t test, and categorical variables (ie, male-to-female ratio, concurrent diarrhea, fever, respiratory illness, breastfeeding status) were compared by chi-square test. P < 0.05 was considered significant. When a variable (eg, CRP, bactericidal antibody titers, proliferation response, or IL-7) was not normally distributed, an appropriate transformation was used to better achieve approximate normality. Analyses were performed on the transformed variables to meet the underlying assumptions of the statistical tests used. When the data could not be normalized, non-parametric analysis (a rank-sum test) was performed. Simple linear and multiple regressions (forward stepwise regression) were used to evaluate the relation between birth weight, nutritional status, and current immunologic variables. Only those variables were tested by the regression model, which showed significant differences between the 2 groups.

### TABLE 1

<table>
<thead>
<tr>
<th>Features</th>
<th>NBW group (n = 66)</th>
<th>LBW group (n = 66)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (wk)²</td>
<td>39.55 ± 0.33</td>
<td>39.33 ± 0.46</td>
<td>0.69</td>
</tr>
<tr>
<td>Weight-for-age (z score)</td>
<td>−1.18 ± 0.05</td>
<td>−2.29 ± 0.07</td>
<td>0.001</td>
</tr>
<tr>
<td>Height-for-age (z score)</td>
<td>−1.22 ± 0.07</td>
<td>−2.09 ± 0.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>32.97 ± 0.14</td>
<td>31.93 ± 0.15</td>
<td>0.001</td>
</tr>
<tr>
<td>Chest circumference (cm)</td>
<td>31.50 ± 0.16</td>
<td>29.50 ± 0.15</td>
<td>0.001</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>47.70 ± 0.17</td>
<td>45.70 ± 0.22</td>
<td>0.001</td>
</tr>
<tr>
<td>Ponderal index (g/cm³)³</td>
<td>17.34 ± 0.13</td>
<td>14.59 ± 0.154</td>
<td>0.001</td>
</tr>
<tr>
<td>Maternal weight (kg)⁴</td>
<td>45.60 ± 0.6</td>
<td>45.78 ± 0.7</td>
<td>0.80</td>
</tr>
<tr>
<td>Maternal MUAC (mm)⁵</td>
<td>228.30 ± 2</td>
<td>227.40 ± 2.4</td>
<td>0.80</td>
</tr>
<tr>
<td>EBF for 3 mo⁶</td>
<td>42/64</td>
<td>48/66</td>
<td>0.49</td>
</tr>
</tbody>
</table>

² MUAC, midupper arm circumference; EBF, exclusive breastfeeding.
Comparison between the 2 groups was done with Student’s t test; P < 0.05 was considered significant.
³ From mother’s last menstrual period.
⁴ Ponderal index = weight/(height²).
⁵ Measurements taken at 3 mo of pregnancy.
⁶ Data on breastfeeding status of 2 children in the NBW group were missing.

### RESULTS

Subjects

Descriptive features of the study children and their mothers at the time of the children’s birth are shown in Table 1. No significant differences were found in gestational age at birth, maternal weight, and maternal MUAC at 3 mo of pregnancy. However, as expected, significant differences were obtained in z scores for weight-for-age and height-for-age, head circumference, chest circumference, length at birth, and ponderal index between the 2 groups at birth. The original birth cohort study showed that poor nutritional status in early pregnancy was the major determinant of LBW in rural Bangladesh (15). One limitation of the study is that, because infants were not followed from birth up to the age of 5 y, data on growth rate, morbidity, vaccination, nutritional status, and other factors were not available, and therefore the effect of those factors on current immune functions and association could not be studied (14). However, data on breastfeeding status were available. Current characteristics of children belonging to the 2 groups are given in Table 2. Proportions of males and females; current age; current weight-for-age, height-for-age, and weight-for-height z scores; body mass index (BMI; in kg/m²); current diarrhea; current fever; and current respiratory illness did not differ significantly between the 2 groups. Because all children included in the study were immunized with vaccines (Bacillus Calmette-Guerin, diphtheria, pertussis, tetanus, oral polio vaccine, and measles) as part of the Expanded Program on Immunization (EPI), vaccination history could not be related to anthropometric measures or other immune functions. No significant differences in the current anthropometric measures and morbidity experiences were found between the exclusively breastfed and nonexclusively breastfed children (duration of exclusive breastfeeding: 3 mo or 6 mo). No differences were found in the current maternal nutritional status, height, or weight or annual family income. According to the growth reference of the National Center for Health Statistics, 58% of the LBW children and 57% of the NBW children were mildly (defined as < −1
TABLE 2
Current baseline features of children born with normal (NBW) or low (LBW) birth weight

<table>
<thead>
<tr>
<th>Features</th>
<th>NBW group (n = 66)</th>
<th>LBW group (n = 66)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mo)</td>
<td>61.80 ± 0.40</td>
<td>60.10 ± 0.48</td>
<td>0.85</td>
</tr>
<tr>
<td>Male/female ratio</td>
<td>32/34</td>
<td>41/25</td>
<td>0.16</td>
</tr>
<tr>
<td>MUAC (mm)</td>
<td>148.86 ± 1</td>
<td>151 ± 2.30</td>
<td>0.30</td>
</tr>
<tr>
<td>Weight-for-age (z score)</td>
<td>−2.20 ± 1</td>
<td>−2.30 ± 0.09</td>
<td>0.68</td>
</tr>
<tr>
<td>Height-for-age (z score)</td>
<td>−1.13 ± 0.10</td>
<td>−1.80 ± 0.60</td>
<td>0.24</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>12.60 ± 0.20</td>
<td>12.5 ± 0.10</td>
<td>0.85</td>
</tr>
<tr>
<td>Current diarrhea (n)</td>
<td>9</td>
<td>14</td>
<td>0.35</td>
</tr>
<tr>
<td>Annual family income ($)</td>
<td>825 ± 104.50</td>
<td>759 ± 132.40</td>
<td>0.69</td>
</tr>
<tr>
<td>Maternal weight (kg)</td>
<td>43.31 ± 0.80</td>
<td>43.30 ± 0.90</td>
<td>0.90</td>
</tr>
<tr>
<td>Maternal height (cm)</td>
<td>155.86 ± 0.50</td>
<td>156 ± 1</td>
<td>0.90</td>
</tr>
<tr>
<td>Maternal MUAC (mm)</td>
<td>237.85 ± 2.50</td>
<td>236 ± 3</td>
<td>0.60</td>
</tr>
</tbody>
</table>

1 MUAC, midupper arm circumference. Continuous variables were compared between the 2 groups by Student’s t test; chi-square test was applied for categorical variables. No significant differences in the baseline features were found between the 2 groups. Current nutritional status in children was assessed through standard anthropometric measures by using the National Center for Health Statistics growth reference. Mild and moderate malnutrition in children was defined as < −1 but ≥ −2 and < −2 but ≥ −3 weight-for-height z scores, respectively. Ponderal index is weight/(height³⁻¹).  
2 ± SD (all such values).  
3 Mothers were asked at the time of the visit by trained field research assistants whether their children had had diarrhea within the past 2–3 d.

TABLE 3
Comparison of immune functions in 5-yr-old children born at normal (NBW) or low (LBW) birth weight

<table>
<thead>
<tr>
<th>Features</th>
<th>NBW group</th>
<th>LBW group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>0.10 (0.0–1.8)</td>
<td>0.65 (0.1–2.6)</td>
<td>0.02</td>
</tr>
<tr>
<td>Bactericidal antibody titers</td>
<td>40 (20–40)</td>
<td>60 (40–160)</td>
<td>0.03</td>
</tr>
<tr>
<td>Proliferation (SI)</td>
<td>15.8 (12.3–86.8)</td>
<td>100.9 (19–112)</td>
<td>0.80</td>
</tr>
<tr>
<td>CD3 lymphocytes (%)</td>
<td>68.3 (64–75)</td>
<td>65.7 (58–69)</td>
<td>0.06</td>
</tr>
<tr>
<td>sjTRECs per 10⁶ PBMCs</td>
<td>0.54 (0.25–0.76) × 10⁵</td>
<td>0.95 (0.42–2.3) × 10⁵</td>
<td>0.05</td>
</tr>
<tr>
<td>IL-7 (pg/mL)</td>
<td>215.7 (0–649.6)</td>
<td>48.4 (0–215.7)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

1 SI, stimulation index; sjTRECs, signal-joint T cell receptor excision circles; PBMCs, peripheral blood mononuclear cells; IL-7, interleukin 7. n in the NBW and LBW groups = 66 and 66, respectively, for C-reactive protein, bactericidal antibody titers, and IL-7; 49 and 51 for proliferation; 45 and 48 for CD3 lymphocytes; and 34 and 34 for sjTRECs. Student’s t test was used for determination of P value. Differences were significant when P < 0.05. Because the variables C-reactive protein, bactericidal titers, SI, and IL-7 were not normally distributed, an appropriate transformation was used to better achieve approximate normality, and analyses were performed on the transformed variables. The bactericidal antibody titer of the serum was expressed as the reciprocal of the last dilution in which no growth was evident by visual inspection.  
2 Median; 25th and 75th percentiles in parentheses (all such values).  
3 SI is the ratio of counts per minute of phytohemagglutinin-stimulated cells to counts per minute of unstimulated cells.  
4 Percentage of pan T CD3 cells in gated lymphocyte cells.

Lymphocyte proliferation response

PBMCs from children were analyzed for the ability of lymphocytes to respond to mitogen (ie, PHA) by proliferation response (incorporation of tritiated thymidine). No significant difference between the 2 groups was found in the SI of lymphocytes to PHA (Table 3). No association was found between lymphocyte proliferation response and current nutritional status by regression analysis.

Proportion of pan T cells in low-birth-weight children

Flow cytometric analysis showed that the proportion of CD3 T cells in the peripheral blood of the LBW group children was not significantly lower than that in the NBW group children; however, the difference showed a trend (P = 0.06) (Table 3). No significant differences between the 2 groups were seen in the percentage of other phenotypic markers or activation markers studied, although a tendency was seen for the NK cell proportions to be lower in the LBW group than in the NBW (data not shown).
Low-birth-weight children

Elevated T cell receptor excision circle concentrations in linear combination.

These variables were forced into the model individually or in a combination.

Neither birth weight nor birth length was associated significantly with CD3 when these variables were not included in the final equation. Neither birth weight nor birth length was associated significantly with CD3, and values for IL-7 and TRECs. None of the variables significantly added to the ability of the equation to predict CD3, and IL-7 values. These variables did not add significantly to the ability of the equation to predict TRECs. Neither birth weight nor birth length was associated significantly with CD3 when these variables were forced into the model individually or in a linear combination.

Elevated T cell receptor excision circle concentrations in low-birth-weight children

Concentrations of TRECs were found to be significantly ($P = 0.05$) higher in the LBW group than in the NBW group (Table 3). Forward stepwise regression was applied to predict TRECs from birth weight, birth length, current nutritional status, and CD3 and IL-7 values. These variables did not add significantly to the ability of the equation to predict TRECs. Neither birth weight nor birth length was associated significantly with TRECs when these variables were forced into the model individually. TRECs values were measured from total PBMCs only, and, because of insufficient blood volumes, cells were not separated into CD4 and CD8 T cells or naïve and memory cells.

Shortened telomeric restriction fragment length in low-birth-weight children

The TRF length in PBMCs ranged from 3.8 to 9.4 kb; the mean length was 7.11 kb in all children. Significantly ($P = 0.02$) lower TRF values were seen in the LBW group than in the NBW group (Figure 2). Shortened telomere length in PBMCs indicated that cells have gone through more cell divisions in the LBW group. Because TRF was measured in total PBMCs, it was not possible to know whether the reduction in TRF involved only lymphocytes or both monocytes and lymphocytes. No association was found between the current nutritional status and the TRF length.

Lower plasma interleukin 7 concentrations in low-birth-weight children

Because the variable was not normally distributed, transformed data were used for statistical analysis. Plasma concentrations of IL-7 were significantly ($P = 0.02$) higher in the NBW group than in the LBW group (Table 3). A forward stepwise regression model was applied to predict IL-7 from birth weight, birth length, current nutritional status, and values for CD3 and TRECs. Birth weight was significantly associated with IL-7 when it was forced into the equation individually ($P = 0.008$), and IL-7 could be predicted significantly from a linear combination of birth weight ($P = 0.028$) and birth length ($P = 0.042$). In this model, birth weight was positively ($\beta = 0.528$) and birth length was negatively ($\beta = -0.318$) associated with IL-7, and the coefficient of determinants ($R^2_{adj}$) obtained from the formula was 0.217. The variables current z scores for weight-for-age, height-for-age, and weight-for-height; MUAC; and TRECs did not significantly add to the ability of the equation to predict IL-7 and were not included in the final equation.

DISCUSSION

The data presented here suggest that LBW children of preschool age have a higher T cell turnover, as assessed by telomere length, TRECs concentrations, and T cell counts than do NBW children. We hypothesized that persistent immune activation in this setting with high levels of exposure to infectious diseases may have resulted in a greater need for thymic production of T cells, thereby generating a void in the T cell compartment.

Several studies have reported that infants with LBW or IUGR or who were SGA have a lower percentage of T or B lymphocytes and lower vaccine-specific IgG responses (2, 3, 21) than do newborn infants with NBW (6, 22–24). However, the persistence of these defects in older children has not been detected (25–27). In one study, schoolchildren born preterm had a significantly lower percentage of CD4+ T cells and lower CD4:CD8 ratios than did children born at term (28). We found that, at a mean age of 5 y, children who were born at full term but with LBW had a lower percentage of CD3 cells in peripheral blood than did NBW children. The lower percentage of CD3+ cells in the LBW group could be a consequence of the limit of division having been met early in life through the accelerated apoptosis of lymphocytes (29). Increased apoptosis could cause increased proliferation in the periphery because lymphocytes strive to “fill the space” in the secondary lymphoid tissues. Indeed, a marked reduction in telomere length in PBMCs was observed in the LBW children; this reduction reflected higher T cell turnover, probably in response to a greater degree of apoptosis. Increased bystander T cell activation and proliferation can occur during HIV infections, and telomere shortening can result (30). Shorter telomeres have been seen in the gastric mucosa of patients with *Helicobacter pylori* infection than in the mucosa of healthy subjects or patients with gastric cancer (31). Premature telomeric loss also has been reported in rheumatoid arthritis (32). However, to our knowledge, this study is the first to show higher T cell turnover in apparently healthy children with LBW, irrespective of current nutritional status.

The role of the thymus as an important indicator of immunologic consequences of undernutrition has been brought into focus by several studies. Measurement of TRECs has been shown to be...
a useful marker of thymic output or thymopoesis. Infants born during the "hungry" (ie, wet) season in rural Gambia were shown to have smaller thymuses and lower TREC concentrations than did infants born in the "harvest" season (11, 12). However, these infants also showed higher lymphocyte count and higher proportion of CD3 T cells. In the current study, the LBW group had higher TREC concentrations in PBMCs, lower percentages of CD3 T cell, and shorter TRF lengths than did the NBW group, albeit lymphocyte proliferation response was similar in the 2 groups. Higher thymic output could have resulted from the heightened demand for T cells in response to high burden of infections in the endemic setting of rural Bangladeshi villages. In HIV-infected subjects, chronic T cell turnover leads to abnormally shortened telomere length (30). The increased clonal exhaustion of cytolytic T cells and reduced cytolytic function in HIV infection have been attributed to replicative senescence. It is conceivable that higher TREC values and shorter telomere length in LBW children than in NBW children may be a consequence of a smaller initial pool of T cells in the thymus, which may lead to earlier exhaustion to maintain homeostatic T cell numbers for rapid replication of memory cells.

IL-7 plays an important role as a regulator of T cell homeostasis. Studies in The Gambia showed markedly higher TREC concentrations in breastfed infants whose mother’s milk had higher IL-7 concentrations than in breastfed infants whose mother’s milk had lower IL-7 concentrations (12). In HIV-infected patients, high IL-7 concentrations are produced in response to T cell depletion (33). The administration of exogenous IL-7 to mice increased the number of TREC-bearing cells (34), although exogenous IL-7 did not always enhance thymic function (35). We found that lower plasma IL-7 concentrations in the LBW children were concurrent with high TREC values. Low IL-7 concentrations in the LBW group may be related to the inability of the periphery to keep up homeostatic proliferation, which requires greater thymic output. An inverse relation between TREC and plasma IL-7 concentrations in HIV-infected children has been reported (36). A positive association of birth weight with plasma IL-7 concentrations also may partly explain low IL-7 concentrations in the LBW children.

In the current study, no association was observed between current nutritional status and any of the markers of immune function, which is consistent with the findings in the Gambian children (37). The current morbidity experiences of the 2 groups (eg, history of diarrhea, respiratory illness, and fever) were not significantly different. Concentrations of CRP found in LBW children were higher than those in NBW children; however, these concentrations were within the "normal" range—ie, they did not indicate the presence of acute inflammation. Numerous large-scale, prospective studies have found that low-grade inflammation, as represented by elevated concentrations of CRP in serum in the range traditionally accepted as normal (<6 mg/L), is an independent predictor of cardiovascular events, especially coronary heart disease (38). In addition, an inverse relation between birth weight and CRP was found: LBW contributed to elevated CRP concentrations in adult life (39) and predicted an elevated risk of cardiovascular events in adulthood (40). In the current study, no association was obtained between CRP concentrations and current nutritional status. However, birth weight had a marked effect on CRP concentrations (P = 0.051) when birth length was included in the multivariate analysis. In the LBW group, CRP concentrations tended to be higher in children with a history of ongoing infections (P = 0.055). Thus, elevated innate markers (ie, CRP and bactericidal activity) and telomere shortening could indicate greater stimulation of the immune system in the LBW group. Heightened innate immunity in the LBW group may be a counterbalance for altered cellular immunity.

A major limitation of the study is that the children were not followed from birth up to the current age, and data on growth rate, morbidity, vaccination and other factors that could potentially confound the results were not available; therefore, the effect of these factors on immune functions and the association could not be studied (14). However, despite these limitations, the findings from the current study suggest that small size at birth may result in altered immune function in later life, which provides further support for the hypothesis that events early in life or in utero may leave a permanent imprint on human immune function. The findings may also reflect a postnatal effect whereby LBW may be a marker for later exposures. It is plausible that the altered immunity may not be manifest in measurable morbidity outcomes unless the person is exposed to repeated infections; however, this validity of that possibility has yet to be seen.

We are indebted to the parents of the subjects for their cooperation and their permission for their children to participate in the study.

RR was responsible for the conception and design of study and for securing funding, implementing the study, supervising the laboratory experiments, compiling and analyzing the data, and drafting the manuscript. DSA (Principal Investigator of the birth cohort study in Matlab from which baseline data of current study subjects and respective mothers at birth were provided) contributed to the implementation of the study. PS processed specimens and performed flow cytometry–associated experiments and fluorescence-activated cell sorter FACS data analyses. SMA contributed to the statistical analysis of the data. GA processed specimens, performed laboratory experiments, and collected data; MY contributed to the implementation of the study and to recruitment of subjects in Matlab. SM contributed to the application of the T cell receptor excision circle method and analysis of the data. GF contributed to the study design and participated in securing funding. None of the authors had a personal or financial conflict of interest.

REFERENCES
Maternal vitamin D intake during pregnancy and early childhood wheezing

Graham Devereux, Augusto A Litonjua, Stephen W Turner, Leone CA Craig, Geraldine McNeill, Sheelagh Martindale, Peter J Helms, Anthony Seaton, and Scott T Weiss

ABSTRACT

Background: Maternal intake of vitamin D in pregnancy is a potentially modifiable but understudied risk factor for the development of asthma in children.

Objective: We investigated whether maternal vitamin D intake in pregnancy is associated with decreased risks of wheezing symptoms in young children.

Design: Subjects were from a birth cohort recruited in utero with the primary objective of identifying associations between maternal diet during pregnancy and asthma and allergies in children. A random sample of 2000 healthy pregnant women was recruited while attending antenatal clinics at the Aberdeen Maternity Hospital, Scotland, at ~12 wk gestation. Maternal vitamin D intake was ascertained from a food-frequency questionnaire completed at 32 wk of gestation. The main outcome measures were wheezing symptoms, spirometry, bronchodilator response, atopic sensitization, and exhaled nitric oxide at 5 y.

Results: Respiratory details through 5 y and maternal food-frequency-questionnaire data were available for 1212 children. In models adjusted for potential confounders, including the children’s vitamin D intake, a comparison of the highest and lowest quintiles of maternal total vitamin D intake conferred lower risks for ever wheeze (odds ratio [OR]: 0.48; 95% CI: 0.25, 0.91), wheeze in the previous year (OR: 0.35; 95% CI: 0.15, 0.83), and persistent wheeze (OR: 0.33; 95% CI: 0.11, 0.98) in 5-y-old children. In addition, lower maternal total vitamin D intakes in pregnancy were also associated with decreased bronchodilator response (P = 0.04). No associations were observed between maternal vitamin D intakes and spirometry or exhaled nitric oxide concentrations.


KEY WORDS Vitamin D intake, pregnancy, wheezing, asthma

INTRODUCTION

Antenatal and early life exposures are increasingly being recognized as determinants of a range of disorders throughout life (1). Although many early postnatal exposures have been studied for their association with asthma, maternal smoking during pregnancy is the only known antenatal modifiable risk factor for reduced lung function (2, 3), wheezing illnesses (3–5), and asthma (5, 6). Maternal diet during pregnancy is a modifiable exposure with the potential to influence the development of asthma and allergies. In 1994, Seaton et al (7) proposed that the recent increases in asthma prevalence could be explained by changes in diet. We subsequently showed, in 2 birth cohorts, that higher maternal dietary vitamin E and zinc intakes in pregnancy are associated with decreased risks of wheezing illnesses and asthma in young children (8–10), which highlights maternal diet as an exposure worth further study.

Vitamin D is both a nutrient and a hormone, and blood concentrations are dependent on dietary intakes and exposure to sunlight. Vitamin D deficiency is well-documented, not only in the elderly but in general populations around the world (11), and current recommended intakes may be inadequate for the maintenance of health (12), particularly in pregnant and lactating women (13, 14). This deficiency may contribute to the increased prevalence of both atopic and autoimmune disorders. Vitamin D and its receptor are important in immune function and development (15) and, therefore, could potentially have a role in the development of asthma and allergies. Initial direct evidence implicating vitamin D in asthma comes from studies showing that vitamin D receptor (VDR) gene polymorphisms are associated with asthma (16, 17) in 2 North American family-based studies. Additionally, recent cross-sectional studies have shown that vitamin D concentrations and vitamin D intakes are associated with lung function level in adults (18) and adolescents (19), respectively.

A separate analysis in a North American birth cohort showed that maternal intakes of vitamin D during pregnancy were inversely associated with wheezing illnesses in 3-y-old children (20). We therefore analyzed data from our Aberdeen cohort to investigate whether this relation is present in addition to those with vitamin E and minerals already reported. Thus, the aim of our study was to investigate the association between maternal vitamin D intake during pregnancy and asthma and wheezing


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illnesses in 5-y-old children in a cohort with distinct demographic and dietary intakes from the North American birth cohort. We also investigated associations between maternal vitamin D intake in pregnancy and lung function, bronchodilator response (BDR), and exhaled nitric oxide in a subset of these children.

SUBJECTS AND METHODS

Study subjects and protocol

Procedures concerning the recruitment of pregnant women and the follow-up of their children were described previously (8, 9). Briefly, 2000 healthy pregnant women were recruited over 19 mo during 1997 and 1999 while attending a hospital antenatal clinic at \( \approx 12 \) wk (median) of gestation (interquartile range (IQR): 11–13 wk). The women were recruited irrespective of their asthma or atopic status and, other than expected slight biases, were representative of the local obstetric population (8). The women were characterized by an interviewer-administered questionnaire, and atopic status was ascertained by skin-prick testing.

At 32 wk of gestation, dietary intake over the preceding 3 mo was assessed by using version 5.4 of the Scottish Collaborative Group Food Frequency Questionnaire (FFQ). Vitamin D intake was expressed as International Units (IU), with 1 IU equating to 0.025 µg cholecalciferol. In 40 women of childbearing age, the Spearman rank correlation coefficients for intakes of vitamin D and calcium derived from this questionnaire and 4-d weighed records were 0.37 \((P < 0.05)\) and 0.75 \((P < 0.001)\) (21).

Assessment of children

Singletons born to the cohort of women were followed up at 2 and 5 y. Six weeks before the children’s second and fifth birthdays, a questionnaire based on the format of the International Study of Asthma and Allergies in Childhood (8, 9) was mailed to all participating families; no more than 2 reminders were sent. Wheeze was defined by an affirmative response to the question, “Has your child had wheezing or whistling in the chest in the last 12 mo?” Similar questions inquired about whether the children had ever wheezed, had ever been asthmatic, and had ever had doctor-diagnosed asthma.

Parents responding to the 5-y questionnaire were invited to participate in assessments of the children’s diet, ventilatory function, and atopic status. Version C1 of the Scottish Collaborative Group FFQ was used to assess the study children’s dietary intake. This FFQ is based on the questionnaire used for the mothers, but was modified for use in children aged 3–5 y by simplifying the response choices and changing the food list and portion sizes to be appropriate for preschool children. In 74 children aged 3–5 y who were recruited from local nurseries, the rank correlation coefficients for intakes of vitamin D and calcium derived from this FFQ and from 4-d nonweighed food diaries were 0.31 \((P = 0.007)\) and 0.38 \((P = 0.001)\) (9).

At 5 y, the responding parents were invited to bring their children to the hospital for an assessment of spirometry, atopic status, and exhaled nitric oxide \((\text{FE}_{\text{NO}})\). The methods used were described previously (9). Briefly, spirometry was measured with a pneumotachograph (Spirotrac IV version 4.22; Vitalograph, Maids Moreton, United Kingdom) with onscreen incentive software. The spirometric values presented were the best from \( \geq 2 \) technically acceptable expiratory maneuvers (22). BDR was expressed as the percentage change in forced expiratory volume in 1 s \((\text{FEV}_1)\) 15 min after inhalation of 400 µg albuterol. BDR was not included in the original study protocol but was introduced for the last 510 children. A NIOX analyzer (Aerocrine, Solna, Sweden) was used to measure \( \text{FE}_{\text{NO}} \) in accordance with international guidelines (23). Measurements of \( \text{FE}_{\text{NO}} \) were not included in the original study protocol but were included in the last 262 assessments after a methodologic study showed that \( \text{FE}_{\text{NO}} \) measurements could be obtained in 65% of children aged 5 y with good reproducibility (24). Atopy was defined as at least one positive skin-prick response (mean weal diameter \( \geq 3 \) mm larger than the negative control) to the allergens cat, timothy grass, egg, and house dust mite (ALK, Hungerford, United Kingdom). The Grampian Research Ethics Committee approved the study, and written parental consent was obtained.

Statistical analysis

The primary outcome variables of interest were the prevalence of wheeze and asthma. In addition to the wheeze outcomes at 2 and 5 y of age, wheezing data for the children at these ages were combined to classify children into wheezing phenotypes analogous to those used in other birth cohorts, namely, never wheezed, early-transient (wheezing at 0–2 y but not at 5 y of age), late-onset (no wheezing at 0–2 and at 5 y of age), and early persistent wheezers (wheezing at 0–2 and at 5 y of age) (25, 26). At 5 y, the secondary outcome variables were \( \text{FEV}_1 \), BDR, \( \text{FE}_{\text{NO}} \), and atopic status. The primary exposure of interest was maternal vitamin D intake. Maternal and children’s dietary and supplemental vitamin D intakes were summed to give a total intake, logarithmically transformed, energy adjusted, and divided into fifths (27). To aid the extrapolation of results to the general population, the quintiles of vitamin D intake were derived from the data for all of the women who completed the FFQ and not merely from those who responded at 2 or 5 y. Univariate associations between outcome variables and explanatory variables were assessed with Mantel-Haenszel odds ratios (OR); multivariate analysis was carried out by using appropriate multivariate regression with adjustment for potentially confounding covariates. Similar analyses related children’s vitamin D intake to respiratory outcomes. Analyses were performed with the use of SPSS version 13.0 (SPSS Inc, Chicago, IL).

RESULTS

Characteristics of mother-infant pairs

Of the 2000 pregnant women recruited, 1751 (87.6%) completed the FFQ. The questionnaire response rates at 2 and 5 y were 1374 (71.4%) and 1253 (65.1%); estimates of maternal vitamin D intake from the FFQ were available for 1335 (97.2%) and 1212 (96.7%) of these groups, respectively, and 1924 singletons were born to the cohort.

Of the 1253 children with symptom questionnaire data at 5 y, dietary data were available for 1120 (89%) and 797 (64%) children who had attended the hospital for assessment. All of the children attempted to perform spirometry; 639 were successful and 478 were able to provide a prebronchodilator \( \text{FEV}_1 \) measurement. Five hundred two children attempted postbronchodilator spirometry; 383 were successful and 238 were able to provide a postbronchodilator measurement. Skin-prick reactivity
and FE\textsubscript{NO} were determined in 700 (56%) and 167 (7%) children, respectively.

The characteristics of the mothers and children responding at 2 and 5 y were described previously (8, 9). Mothers responding to the 2- and 5-y questionnaire were less likely to smoke, were older, were of higher socioeconomic status (SES), were less likely to have wheezed or to have had asthma, and had slightly higher vitamin D intakes than did the women who failed to respond (Table 1) (8, 9). The participating children were slightly larger at birth, were more likely to have had a cesarean delivery, were more likely to have been breastfed (8, 9). The mothers and children who attended the hospital for the assessment of spirometry, FE\textsubscript{NO}, and atopic status were representative of those responding to the questionnaire (9).

The characteristics of the mothers and children at recruitment and delivery and at the follow-up of the children at 2 and 5 y are outlined in Table 2. For more details of the cohort characteristics, see Table A under “Supplemental data” in the current online issue at www.ajcn.org. Maternal total vitamin D intake was negatively associated with smoking and was positively associated with age, SES, breastfeeding of child, other children in the home at 5 y, use of vitamin D supplements, and intakes of vitamin E, zinc, and calcium. Total vitamin D intake by the children was positively associated with maternal total vitamin D intake (rank correlation coefficient = 0.16, P < 0.001). No independent significant associations were observed between maternal vitamin D intake and wheezing symptoms in the children at 2 y of age.

### Maternal vitamin D intake and symptoms in children aged 5 y
Maternal total vitamin D intake from the diet and supplements was negatively associated with the symptoms “ever wheeze”, “wheeze in the previous year,” and “persistent wheeze” at 2 and 5 y of age (Table 3) but not with asthma in children aged 5 y (OR per quintile of maternal energy-adjusted vitamin D intake 0.99, 95% CI: 0.83, 1.17, P = 0.98). We previously reported in this cohort that maternal vitamin E and zinc intakes are associated with 5-y outcomes; however, the associations with maternal total vitamin D intake remained significant even after adjustment for maternal vitamin E and zinc intakes. Separate analyses in which maternal smoking status during pregnancy was replaced with parental smoking status or with the number of smokers in the 5-y-old child’s house (24.5% of 5-y-olds were from a smoking household) did not change the nature or the strength of the associations with vitamin D. The associations between maternal total vitamin D intake and 5-y outcomes were independent of total vitamin D intake by the children.

Associations similar to those seen for total intakes were seen for maternal dietary vitamin D intake during pregnancy. No associations between children’s symptoms at 5 y and maternal calcium intake were observed.

### Maternal vitamin D intake and atopy and FE\textsubscript{NO} in children aged 5 y
Maternal total and dietary vitamin D intakes were not associated with atopic sensitization (700 children) or with FE\textsubscript{NO} (167 children).

### Maternal vitamin D intake and spirometry in children aged 5 y
Maternal total and dietary vitamin D intake were not associated with prebronchodilator FE\textsubscript{V_0.5}, FE\textsubscript{V_0.75}, FE\textsubscript{V_1}, FE\textsubscript{V_25-75}, FE\textsubscript{V50}, PEF, and FVC in univariate or multivariate analyses. The median BDR in 238 children was 4.4% (interquartile range: 0-8.4). The distribution of BDR was normal for this population. In a univariate analysis, BDR was 3.6% in the 39 children whose

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**Table 1**

<table>
<thead>
<tr>
<th>Vitamin D</th>
<th>Response at 2 y</th>
<th>Response at 5 y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Responder</td>
<td>Nonresponder</td>
</tr>
<tr>
<td></td>
<td>(n = 1335)</td>
<td>(n = 416)</td>
</tr>
<tr>
<td>Median intake (IU/d)(^1)</td>
<td>128 (99–170)</td>
<td>131(^2) (101–175)</td>
</tr>
<tr>
<td>5th–95th Centiles (IU/d)</td>
<td>67–445</td>
<td>68–468</td>
</tr>
</tbody>
</table>

\(^1\) North American Adequate Intake for pregnant women = 200 IU/d. Interquartile range in parentheses.

\(^2\) P < 0.001 (Mann-Whitney U test).

---

**Table 2**

Characteristics of mother-infant pairs at recruitment and delivery and of the children responding at 5 y\(^1\)

<table>
<thead>
<tr>
<th>Mothers (n = 1751)</th>
<th></th>
<th>Children (n = 1751)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Median energy-adjusted intake (IU/d)(^2)</td>
<td>128</td>
<td>Boys (%)</td>
<td>51.2</td>
</tr>
<tr>
<td>Interquartile range (IU/d)</td>
<td>103–165</td>
<td>Mean birth weight (g)</td>
<td>3099 (2974, 3233)</td>
</tr>
<tr>
<td>5th–95th percentile (IU/d)</td>
<td>67–445</td>
<td>Ever breastfed (%)</td>
<td>72.0</td>
</tr>
<tr>
<td>Use of vitamin D supplements (%)</td>
<td>10.5</td>
<td>Children at 5 y (n = 1253)</td>
<td></td>
</tr>
<tr>
<td>Use of any vitamin supplements (%)</td>
<td>45</td>
<td>Smoker in home (%)</td>
<td>24.5</td>
</tr>
<tr>
<td>Geometric mean calcium intake. (mg/d)</td>
<td>1232 (1209, 1255)</td>
<td>One adult smoker in home (%)</td>
<td>16.2</td>
</tr>
<tr>
<td>Maternal age at enrollment (y)</td>
<td>29.2 (28.9, 29.4)</td>
<td>≥2 Smokers in home (%)</td>
<td>8.3</td>
</tr>
<tr>
<td>Partner of nonmanual social class (%)</td>
<td>57.9</td>
<td>Median energy-adjusted vitamin D intake (IU/d)</td>
<td>47</td>
</tr>
<tr>
<td>Maternal smoking during pregnancy (%)</td>
<td>26.7</td>
<td>Interquartile range (IU/d)</td>
<td>35–88</td>
</tr>
<tr>
<td>Ever asthma (%)</td>
<td>16.1</td>
<td>5th–95th percentile (IU/d)</td>
<td>22–242</td>
</tr>
<tr>
<td>Atopic sensitisation (%)</td>
<td>35.8</td>
<td>Use of vitamin D supplements (%)</td>
<td>24.1</td>
</tr>
</tbody>
</table>

\(^1\) 95% CI in parentheses.

\(^2\) North American Adequate intake for pregnant women = 200 IU/d.
Children’s vitamin D intake and symptoms at 5 y of age

No significant associations were observed between the total and dietary vitamin D intakes of children and respiratory symptoms, atopic sensitization, spirometry, or FE\textsubscript{NO}.

TABLE 4
Results of linear regression analysis relating bronchodilator response (%) to maternal daily total vitamin D intake during pregnancy (IU/d).^1

<table>
<thead>
<tr>
<th>Quintile of energy-adjusted maternal vitamin D intake</th>
<th>Median energy-adjusted intake (IU/d)</th>
<th>P for trend\textsuperscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n = 213)</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>2 (n = 246)</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>3 (n = 237)</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>4 (n = 261)</td>
<td>157</td>
<td></td>
</tr>
<tr>
<td>5 (n = 255)</td>
<td>275</td>
<td></td>
</tr>
<tr>
<td>5th–95th percentile (IU/d)</td>
<td>46–92</td>
<td></td>
</tr>
<tr>
<td>95th percentile</td>
<td>117–139</td>
<td></td>
</tr>
<tr>
<td>90th percentile</td>
<td>142–182</td>
<td></td>
</tr>
<tr>
<td>75th percentile</td>
<td>189–751</td>
<td></td>
</tr>
</tbody>
</table>

Univariate and multivariate analyses of total maternal vitamin D intake and likelihood of wheezing symptoms in children aged 5 y\textsuperscript{1}

<table>
<thead>
<tr>
<th>Quintile of energy-adjusted maternal vitamin D intake</th>
<th>Median energy-adjusted intake (IU/d)</th>
<th>P for trend\textsuperscript{2}</th>
</tr>
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<td>75th percentile</td>
<td>189–751</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION
Our analyses showed that low maternal dietary and total vitamin D intakes during pregnancy are associated with increased wheezing symptoms in children at the age of 5 y. These associations were independent of maternal smoking status and maternal intakes of vitamin E, zinc, calcium, and vitamin D by the 5-y-old children.

Vitamin D is important for the regulation of calcium homeostasis, bone formation, and resorption. However, VDRs (28, 29) and vitamin D metabolic enzymes (11, 30) have been identified in many tissues other than bone and the intestine, which suggests involvement in the metabolism and function of many cell types. Specifically, VDRs are expressed in cells of the immune system, such as T cells (31), activated B cells (32), and dendritic cells (33). Indeed, vitamin D has been linked with a diverse group of disorders characterized by immunologically mediated inflammation, such as type 1 diabetes mellitus (34, 35), multiple sclerosis (36), and rheumatoid arthritis (37). Additionally, vitamin D has been implicated in the susceptibility to mycobacterial (38, 39) and HIV (40) disease and with antimicrobial innate immune responses (41). With regard to the development of asthma and allergies, experimental models of asthma have shown that vitamin D may alter the balance between T helper subset 1 and T helper subset 2 cell cytokine secretion (42, 43). Reduced secretion of the T helper subset 1 cell cytokines interleukin (IL) 2 and interferon (IFN) γ (43, 44) and an increase in the T helper subset 2 cell cytokine IL-4 (45, 46) have been observed after treatment with 1,25-dihydroxyvitamin D. In contrast, Pichler et al (47) showed that in human CD4\textsuperscript{+} and CD8\textsuperscript{+} cord blood cells, vitamin D inhibits not only IL-12–generated...
IFN-γ production, but also suppresses T helper subset 2 cell–related IL-4 and IL-4–induced expression of IL-13. Although seemingly contradictory, it is possible that the effects of vitamin D on these cells are dependent on the timing of exposure (ie, prenatal compared with postnatal); thus, the response to vitamin D exposure of naïve T cells in the fetus or neonate may differ from that of mature cells (48). Because vitamin D deficiency has been documented in populations around the world, we speculate that this may contribute to the rise in prevalence of both T helper subset 1 cell and T helper subset 2 cell diseases.

Vitamin D has been linked to fetal lung development in animal models (49, 50), and higher vitamin D concentrations and intakes are associated with higher lung function in adults (18) and adolescents (19), respectively. Although we did not find any association between maternal vitamin D intake and lung function at 5 y, we did see a positive association with BDR in a subset of the children. This association should be interpreted with caution because, although the children able to achieve a post-BDR were representative of those attending for clinical evaluation, they were relatively small in number (n = 238). The positive association with BDR appears contradictory to our wheeze findings; however, a greater BDR in early childhood asthmatics has been shown to predict higher $FEV_1$ values after 4 y (51), which suggests that a larger BDR in childhood may be a marker of greater potential lung growth as the child gets older. Further follow-up of the cohort is needed to clarify this association.

Initial evidence implicating vitamin D in asthma and allergy development came from studies of genetic associations in humans. Significant associations between polymorphisms in the VDR gene with asthma have been reported in 2 family-based studies of North American subjects (16, 17). However, 2 subsequent German studies found no significant associations (52, 53). In addition to genetics, evidence indicates that vitamin D may have a therapeutic role in asthma by enhancing responsiveness to glucocorticoids for induction of IL-10 (54). A birth cohort from Northern Finland has shown that vitamin D supplementation in the first year of life increased the risk of asthma and atopy at 31 y of age (55). However, this study did not assess maternal or childhood vitamin D intakes and did not assess childhood asthma and atopy. The results of the present study are consistent with only one other study (20) to date that has assessed maternal vitamin D intakes during pregnancy; both studies indicated that childhood vitamin D intake is not associated with recurrent wheezing in childhood and that prenatal mechanisms probably underlie the effect of vitamin D.

The original study population of 2000 pregnant women was very similar demographically to the local obstetric population (8), but there has been some loss to follow-up with time (9). Of particular concern were the lower response rates of women with a lower SES, who were more likely to smoke during pregnancy and to have lower intakes of vitamin D during pregnancy. Accordingly, several measures of smoking exposure (maternal smoking status during pregnancy and number of smokers in the household at age 5 y) and SES status (maternal age of leaving full time education, paternal social class, ad deprivation score based on area of residence) were included in the regression analysis; however, the inclusion of these measures had a minimal effect on the association between maternal vitamin D intake and wheezing outcomes. Although the evidence indicated response biases typical of this type of study, it is unlikely that these biases accounted for our observations because the nature of the biases would be to weaken the observed negative associations rather than to augment them. (See “Supplemental data” in the current online issue at www.ajcn.org for a full discussion of these potential biases.)

At the time of inception of the study, it was not standard practice to recommend universal vitamin supplementation during pregnancy, thus, only 45% of the women were taking any vitamin supplements, and 10.5% were taking supplements containing vitamin D. The vitamin D intakes of the women participating in the study were slightly higher ($\bar{x}$: 137 IU/d) than the average vitamin D intake by women in the United Kingdom ($\bar{x}$: 112 IU/d) reported by the UK National Diet and Nutrition Survey (56), which possibly reflects the enrichment of our study sample by women of a higher SES. These means are still below the current recommended intakes for pregnant women in North America (Adequate Intake = 200 IU/d) (57).

The North American study (20) and the present study have identified similar associations in 2 geographically disparate areas (Boston, MA, 42 °N; Aberdeen, Scotland, 57 °N) in 2 populations that differ demographically. When compared with their North American counterparts, mothers in the present study had a lower vitamin D intake (137 compared with 548 IU/d in the North American study), were younger, were more likely to smoke during pregnancy, were less likely to have a degree, and were almost universally white (98%), which reflected the local population. Although the demonstration of similar associations in 2 distinct populations increases confidence in the validity and biological relevance of the associations, we cannot entirely eliminate the possibility that the observed associations are a consequence of residual confounding by factors associated with a higher SES and a healthy lifestyle.

In the United Kingdom, margarine is fortified with vitamin D and common sources of dietary vitamin D for UK women are fish (25%), meat and meat products (22%), cereals and grains (21%), spreads (17%), and eggs (9%). There is some overlap with the food groups that are sources of dietary vitamin E intake, spreads (18%), cereals and grains (17%), potato products (13%), vegetables (13%), meat and meat products (11%), fish (5%), and eggs (3%) (52). It seems unlikely that the associations between maternal vitamin D intakes and childhood wheezing could be a consequence of confounding by maternal vitamin E intake, because the associations with vitamin D persisted after adjustment for maternal vitamin E intake. Although we did not observe a statistically significant association between maternal vitamin D intakes and childhood asthma, we did find an inverse association with asthma treatment (data not shown). Thus, further follow-up of this cohort will clarify this association.

In summary, we report an inverse association between maternal vitamin D intake in pregnancy and risk of recurrent wheezing in 5-y-old children. Our results are of great public health significance because they could lead to relatively low cost interventions of vitamin D supplementation that would have a large effect on the future prevalence of asthma in children.

GD, AS, PJH, and GM designed the overall study, obtained funding, and provided critical reviews of the manuscript. GD defined the variables, analyzed the data, and wrote and revised the manuscript. AAL conceived and designed the analysis plans, assisted in the data analysis, and wrote and revised the manuscript. SWT supervised the clinical follow-up of the children, analyzed the spirometry and $FEV_1$ data, and provided critical reviews. LCAC managed the study database, ensured quality control of the data, validated the children’s FFQ, and provided critical reviews of the manuscript. GM provided nutritional advice and critical reviews of the manuscript. SM
recruited the cohort of mothers, conducted the follow-up of the children at 2 y, and provided critical reviews. STW helped conceive the analysis plans, provided critical reviews, and helped revise the manuscript. The authors had no conflicts of interest to report related to the data in the manuscript.

REFERENCES

13. Hollis BW, Wagner CL. Vitamin D requirements during lactation: high-dose maternal supplementation as therapy to prevent hypovitaminosis D for both the mother and the nursing infant. Am J Clin Nutr 2004;80(suppl):1752S–8S.
Hypovitaminosis D in British adults at age 45 y: nationwide cohort study of dietary and lifestyle predictors1–3

Elina Hyppönen and Chris Power

ABSTRACT

Background: Increased awareness of the importance of vitamin D to health has led to concerns about the prevalence of hypovitaminosis D in many parts of the world.

Objectives: We aimed to determine the prevalence of hypovitaminosis D in the white British population and to evaluate the influence of key dietary and lifestyle risk factors.

Design: We measured 25-hydroxyvitamin D [25(OH)D] in 7437 whites from the 1958 British birth cohort when they were 45 y old.

Results: The prevalence of hypovitaminosis D was highest during the winter and spring, when 25(OH)D concentrations <25, <40, and <75 nmol/L were found in 15.5%, 46.6%, and 87.1% of participants, respectively; the proportions were 3.2%, 15.4%, and 60.9%, respectively, during the summer and fall. Men had higher 25(OH)D concentrations, on average, than did women during the summer and fall but not during the winter and spring (P = 0.006, likelihood ratio test for interaction). 25(OH)D concentrations were significantly higher in participants who consumed vitamin D–fortified margarine rather than those who did not (P < 0.0001 for both) but were not significantly higher in participants who consumed vitamin D–fortified margarine than in those who did not (P = 0.10). 25(OH)D concentrations <40 nmol/L were twice as likely in the obese as in the nonobese and in Scottish participants as in those from other parts of Great Britain (ie, England and Wales) (P < 0.0001 for both).

Conclusion: Prevalence of hypovitaminosis D in the general population was alarmingly high during the winter and spring, which warrants action at a population level rather than at a risk group level.


KEY WORDS 25-Hydroxyvitamin D, vitamin D status, vitamin D supplements, vitamin D deficiency, seasonality, fortified food, population studies, Great Britain

INTRODUCTION

The thinking about the actions of vitamin D have made an important shift during the past 10 y. In addition to its well-established role in the regulation of calcium metabolism, the active form of vitamin D has been shown to have antiproliferative and immunomodulatory effects that are thought to influence the development of several serious conditions, including diabetes, cardiovascular disease, and cancer (1–6). Vitamin D is a nutrient that functions as a hormone precursor, and wide-ranging health effects are supported by the presence of vitamin D receptors in several cell types and tissues of the body (eg, lymphocytes and monocytes, brain, heart, pancreas, intestine, and placenta; 2). With this increasing knowledge, a reconsideration of the cutoffs for adequate vitamin D status has occurred. 25-Hydroxyvitamin D [25(OH)D] is the best available indicator for vitamin D status (7), and concentrations ≥25 nmol/L are sufficient to prevent the severe hypovitaminosis D that leads to softening of bone tissue, which manifests as rickets in children and as osteomalacia in adults (1, 4, 8). Furthermore, it is now understood that even less severe forms of hypovitaminosis D have short- and long-term health implications, and accordingly, in a recent consensus statement, concentrations of ≥75 nmol/L were identified as necessary for optimum bone health (9). This cutoff was based on a threshold required for a range of functional outcomes, including maximal suppression of circulating parathyroid hormone, greatest calcium absorption, and highest bone mineral density.

Concerns exist that hypovitaminosis D may be common in many parts of the world; some discussion has been held of possible epidemics in Western populations, and calls have been heard for screening of vitamin D status in routine health care surveillance (2). We aimed to evaluate the magnitude of hypovitaminosis D as a public health problem in Great Britain (ie, England, Scotland, and Wales), because several reasons exist for suspicion that it is particularly prevalent there. Skin synthesis of vitamin D (the major source of the vitamin) is likely to be affected by Western lifestyles, which increasingly involve working indoors during daylight hours, and this may be particularly important when combined with residence in northern latitudes and a cloudy climate (10). Moreover, dietary intake of vitamin D may be low in Great Britain, where vitamin D fortification is mandatory only for margarine. This situation contrasts with that in other countries, such as the United States and Canada, where milk is also fortified (11). With the exception of oily fish, most foods naturally contain very little vitamin D (2, 3). Intake in the form of supplements is limited to the availability of over-the-counter products. Finally, the high prevalence of obesity in Great Britain

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(12), a well-known risk factor for hypovitaminosis D (13, 14), may adversely affect vitamin D status in the population.

Alarming high rates of hypovitaminosis D have been found in ethnic minorities living in Great Britain (15–17). Case reports document a reemergence of rickets in infants (18), children (19), and adolescents (20); in addition, hypovitaminosis D is common in the elderly, particularly those living in institutions (21, 22). We measured serum 25(OH)D in 2002–2004 in a nationwide sample of whites (aged 45 y) who are participants in the 1958 birth cohort study. Our aims here were to report current prevalence rates and to examine seasonal, demographic, and lifestyle influences on hypovitaminosis D.

SUBJECTS AND METHODS

Subjects

Participants are from the 1958 British birth cohort; all were born in England, Scotland, or Wales during the same week of March 1958 (n = 16 751; 23, 24). Cohort members were most recently contacted between September 2002 and April 2004 (24). The target population for this survey consisted of 12 069 persons currently living in Great Britain. Seventy-eight percent (n = 9 349) of participants completed questionnaires, and 7 591 (81%) also provided a blood sample, which was used to determine their vitamin D status. The cohort is mainly white (98%); immigrants born during the target week were included in the birth cohort study only up to 1974. The current study is restricted to whites (including British, ethnically Irish living in Great Britain, and other whites); persons of other ethnic origins (n = 154) were excluded.

Written informed consent for the use of information in medical studies was obtained from the cohort members. The 2002–2004 survey was approved by the South-East Multi-Centre Research Ethics Committee.

Methods

Weight and standing height at age 45 y were measured without shoes and in light clothing by the nurse using scales and a stadiometer. Body mass index (BMI; in kg/m²) was calculated, and obesity was defined as BMI ≥ 30. The season in which the blood sample was obtained was classified as winter (December through February), spring (March through May), summer (June through August), and fall (September through November). Geographic location was based on current region of residence, which, for presentation and analyses of north-south gradient, was grouped as follows (denoted by using the standard British terminology for regional boundaries in Great Britain): South (South East, South West, and Greater London), Middle (East Anglia, Midlands, and Wales), North (North West and Yorkshire and the Humber), and Scotland. Socioeconomic status (SES) was assessed by using the Registrar General’s occupational classification based on current or most recent occupation at age 42 y and categorized as I and II (professional and managerial), III (skilled) nonmanual, III (skilled) manual, and IV and V (partly skilled and unskilled). Persons who were institutionalized, retired, or unemployed over a long period were classified separately (n = 319; 4.3%).

Information on selected dietary and lifestyle influences on 25(OH)D was collected by using a structured food-frequency questionnaire (FFQ). Frequencies of dietary intake were reported as never, occasionally, <1 d/wk, 1–2 d/wk, 3–6 d/wk, 1 time/d, 1–4 times/d, and >4 times/d. Consumption of oily fish (eg, salmon, trout, and mackerel) was classified as weekly, less than weekly, and never, and margarine use was classified as daily, weekly, and less than weekly. Vitamin D fortification of margarine (7.05–8.82 µg/100 g) is mandatory in the United Kingdom (25). Participants reported their use of cod liver or fish oil or other supplements containing vitamin D [typical vitamin D concentration in over-the-counter supplements sold in the United Kingdom (D₃ or D₂) is 200 IU]. The usual time per day spent outdoors in daylight hours during the previous month was reported as no time, <15 min, 15–30 min, 30–60 min, 1–2 h, 3–4 h, and >4 h.

Measurement of vitamin D status

25(OH)D was measured by using automated application of an enzyme-linked immunosorbent assay (IDS OCTEIA Elisa; IDS, Bolton, United Kingdom) and an analyzer (BEP 2000; Dade-Behring, Milton Keynes, United Kingdom) with sensitivity of 5.0 nmol/L, linearity ≤ 155 nmol/L, and intraassay CV 5.5–7.2% (26, 27). The heterogeneity of 25(OH)D concentrations measured by different assay methods is well known (7, 28). To apply previously recommended cutoffs for hypovitaminosis D, 25(OH)D concentrations were standardized according to the mean of the values found by the Vitamin D External Quality Assurance Survey (DEQAS) of >100 laboratories around the world (9, 28). Standardization was based on quality-control data compared on 5 occasions during the study period; evaluations were conducted at the start of the fieldwork and then at 3-mo intervals. Hypovitaminosis D was defined by using 3 thresholds for 25(OH)D concentrations: <25, <40, and <75 nmol/L. Cutoffs were selected on the basis of 1) concentrations sufficient to prevent rickets and osteomalacia (ie, <25 nmol/L) (1, 4, 8); 2) a lower reference concentration suggested to reflect the need for vitamin D supplementation according to most laboratories carrying out vitamin D assays (ie, <40 nmol/L) (3, 28); and 3) current judgment as to the concentration required for optimal bone health (ie, <75 nmol/L) (4, 9).

Statistical analysis

Natural log transformation was used to achieve normal distribution for 25(OH)D. Log-transformed values were used in calculating geometric means and for determining the outcome in linear regression analyses. For descriptive purposes, mean 25(OH)D concentrations and the proportion of persons with hypovitaminosis D are presented after standardization by sex and season. Comparisons of seasonal and demographic factors with dietary and lifestyle indicators of vitamin D status were analyzed by chi-square test and by nonparametric test for trend in the case of ordinal categorized variables. P < 0.05 was considered significant. Log likelihood ratio tests (LRT) and LRTs for trend were used to determine associations with serum 25(OH)D. All
analyses of serum 25(OH)D were done by using linear regression after adjustment for sex and month of measurement.

Influences on hypovitaminosis D were evaluated by logistic regression after adjustment of all models for sex and month of measurement. Odds ratios and corresponding 95% CIs were used to describe the influence of demographic, dietary, and lifestyle indicators on the risk of hypovitaminosis D. LRTs and LRT trend tests were used to test the significance of each factor in the models. The first model included adjustment for sex and month of measurement only. The fully adjusted model included, in addition, all other indicators of hypovitaminosis D. Logistic regression analyses were repeated by using the 3 thresholds for vitamin D supplementation, intake of oily fish, margarine consumption, use of sun protection, time spent outdoors, or watching TV or using a PC. Analyses were repeated for the sample with complete data, and results were unaffected by the treatment of missing information unless otherwise indicated. Results are presented with imputed data. Statistical analyses were carried out by using STATA software (version 9.1; Stata Corp, College Station, TX), and maps were constructed with the use of EPIMAP software (version 3.3.2; Epi Info, Atlanta, GA).

RESULTS

Demographic characteristics and associations with supplement use and time spent outdoors are presented in Table 1. Women and nonobese participants (BMI < 30) were significantly more likely to use vitamin D supplements and to spend less time outdoors than were others. Variations in the frequency of oily fish consumption were similar to those observed for supplement use: compared with others, fish consumption was significantly more frequent in females, the nonobese, participants living in Southern England, and those in SES classification I and II (P ≤ 0.001 for all comparisons, chi-square test). Women were significantly more likely to use sun protection than were men: 70% and 49%, respectively, classified their use as “often” and 5% and 15%, respectively, classified their use as “rarely or
never” \((P < 0.0001, \text{chi-square test})\). SES differences were seen in the use of sun protection: the proportion of participants reporting use “often” and “rarely or never,” respectively, was 63% and 8% in SES classification I and II, 67% and 7% in SES classification III (skilled) nonmanual, 50% and 15% in SES classification III (skilled) manual, 57% and 12% in SES classification IV and V, and 57% and 14% in the unclassified group \((P < 0.0001, \text{chi-square test})\). Variation in sun protection by BMI was modest: “often” and “rarely or never” in 58% and 12%, respectively, of the obese compared with 61% and 9%, respectively, of the others \((P < 0.03, \text{chi-square test})\), whereas no significant variations were seen by region \((P > 0.07, \text{chi-square test})\).

Serum 25(OH)D concentrations peaked in September and were at their lowest from January through April (Figure 1). Month of blood sampling was the strongest predictor; it explained 21.5% of the variation in 25(OH)D \((P < 0.0001, \text{LRT})\). The association between 25(OH)D and sex varied by month of measurement; concentrations tended to be lower in women during the summer and fall, whereas no consistent monthly sex differences were apparent during the winter and spring (December through May; Figure 1). The use of vitamin D supplements and the consumption of oily fish but not (fortified) margarine showed the expected associations with 25(OH)D (Figure 2). Time spent outdoors was strongly associated with 25(OH)D during the summer and fall, but no association was apparent during the winter months \((P < 0.0001, \text{LRT interaction})\).

Because the 25(OH)D concentration was largely predicted by the month of blood sampling, the prevalence of hypovitaminosis D, as shown in Table 2, is stratified by sex. Hypovitaminosis D at all thresholds was more common in women than in men during the summer and fall, whereas, during the winter and spring, women were overrepresented among those with 25(OH)D \(< 25 \text{nmol/L and } \geq 75 \text{nmol/L}. The prevalence of hypovitaminosis D was markedly higher in the obese than in the others in all severity groups and regardless of season. There was a significant north-south gradient in the prevalence of hypovitaminosis D in all severity groups (Table 2). Regional variation in the prevalence of 25(OH)D concentrations \(< 40 \text{nmol/L during the 4 seasons is represented in Figure 3.}\)

As reported in Table 2, the association between sex and hypovitaminosis D was dependent on season. Women had a risk of having 25(OH)D \(< 25 \text{nmol/L during the summer or fall twice that of men, whereas women’s risk of concentrations } < 40 \text{nmol/L and } < 75 \text{nmol/L were only 40% greater than that of men after full adjustment for available background indicators (LRT } P < 0.0001 \text{ for all comparisons adjusted for month of measurement, skin color, obesity, region, SES, supplement use, fish consumption, time spent outdoors, sun protection and time spent watching TV or using a PC). During winter, the only threshold at which the risk of hypovitaminosis D was increased in women was the threshold of } < 25 \text{nmol/L}(P = 0.001; \text{adjusted OR: 1.50; LRT}). Persons who were obese or who lived in Scotland had a risk of 25(OH)D concentrations \(< 40 \text{nmol that was twice that of nonobese persons or those who did not live in} \)

![FIGURE 1. Geometric mean (95% CI) monthly variation in serum 25-hydroxyvitamin D [25(OH)D] concentrations in men (□; \(n = 3,725\)) and women (□; \(n = 3,712\)) in the 1958 British birth cohort at age 45 y. The interaction between sex and month was significant \((P = 0.02, \text{linear regression analyses on log 25(OH)D)}\). \(n\) per sex and month ranged from 17 to 340: 98 in December 2003 for women and \(< 100\ for both sexes in December 2002 (n = 40 M, 37 F), January 2004 (n = 95 M, 75 F), February 2004 (n = 58 M, 70 F), and March 2004 (n = 22 M, 17 F).}]}
Scotland (Table 3). The strength of the association with vitamin D supplementation and margarine consumption varied according to the severity of hypovitaminosis D. Use of vitamin D supplements was associated significantly more strongly with the 2 lowest thresholds (fully adjusted OR for 25 \( \text{nmo/L} \): 0.33; 95% CI: 0.2, 0.5; OR for 40 nmol/L: 0.36; 95% CI: 0.3, 0.4) than with a threshold of 75 nmol/L (OR: 0.52; 95% CI: 0.5, 0.6). Margarine consumption was not associated with 25(OH)D concentrations >75 nmol/L and was not robustly associated with those <40 nmol/L (Table 3), but the risk of a concentration <25 nmol/L was less in cohort members who consumed margarine daily (adjusted OR: 0.69; 95% CI: 0.6, 0.8) or weekly (adjusted OR: 0.68; 95% CI: 0.5, 0.9) than in those with less frequent consumption (adjusted LRT for trend, \( P = 0.003 \)). As suggested by Figure 2, time spent outdoors during winter did not affect the risk of hypovitaminosis D (\( P = 0.18 \)), however, there was a strong linear association for all severity groups at other times of the year (adjusted LRT for trend, \( P < 0.0001 \) for all comparisons).

**DISCUSSION**

Previous studies have highlighted the emerging problems of hypovitaminosis D in the elderly in Great Britain (21, 22) and ethnic minority groups (15–17). Our results suggest that the problem is widespread also in middle-aged British whites. It is striking that nearly half of the population had 25(OH)D concentrations <40 nmol/L during the winter and spring (3, 28); this showed that, for part of the year at least, the problem is not restricted to high-risk groups. With the use of the higher cutoff from a recent consensus on optimal status (ie, 75 nmol/L) (9), it is disturbing from the viewpoint of future bone health that nearly 90% of the current study population was affected by hypovitaminosis D during the winter and spring, and 60% had suboptimal concentrations year-round.

Old age is a well-established risk factor for hypovitaminosis D (2) and as expected our middle-aged population compares favorably to Britons aged >65 y (21, 22). It is reassuring that our results are confirmed by statistics for 35–49-y-olds in the National Diet and Nutrition survey (mean concentration for both men and women: 48 nmol/L) (30). Nonetheless, the mean 25(OH)D concentration was lower and the prevalence of hypovitaminosis D higher in our study than in the general adult population in Canada or the United States. For example, in a study from Calgary, Canada (latitude 51°N) 20% of participants had 25(OH)D concentrations <40 nmol/L during the winter (31), whereas, in the current study, that proportion was 46%. In the 2

![FIGURE 2. Geometric mean (95% CI) 25-hydroxyvitamin D [25(OH)D] concentrations by dietary and lifestyle indicators, standardized by sex and season.](image-url)
<table>
<thead>
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<th>Characteristics and season</th>
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<th>Average</th>
<th>&lt;25 nmol/L (n = 148/439)</th>
<th>&lt;40 nmol/L (n = 705/1327)</th>
<th>&lt;75 nmol/L (n = 2794/2481)</th>
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<td>nmol/L</td>
<td>%</td>
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<td>Summer and fall</td>
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<td>60.9</td>
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<td>88.7</td>
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<td>17.1</td>
<td>46.3</td>
<td>85.4</td>
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<tr>
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<td>0.72</td>
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<td></td>
</tr>
<tr>
<td>Men</td>
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<td>2.2</td>
<td>13.4</td>
<td>58.1</td>
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<td>46.9</td>
<td>87.9</td>
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<td>classifications)</td>
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<td>86.3</td>
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<td>0.008</td>
<td>0.17</td>
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</table>

1 All means and proportions are standardized by sex and season (winter and spring = December through May; summer and fall = June through November). 
2 P values for differences in 25(OH)D or prevalence of hypovitaminosis D from log likelihood ratio tests or log likelihood ratio trend tests as indicated. Sex, obesity, and socioeconomic status were distributed equally across seasons (P ≥ 0.41 for all comparisons, chi-square test). A small difference was found between regions of interviews carried out in the summer and fall: 54% in southern England compared with 64% in the Midlands and 65% in northern England compared with 64% in Scotland (P < 0.0001, chi-square test). Season modified the association between sex and 25(OH)D (P = 0.0006) and socioeconomic status and 25(OH)D (P = 0.01); no interaction was observed for other factors. 
3 The number of subjects with hypovitaminosis D in December through May and June through November. 
4 Defined as BMI (in kg/m²) ≥30. Unknown: December through May, n = 47; June through November, n = 52. P value from test including data for full year. 
5 P value from test including data for full year. 
6 I and II, professional or managerial; III, nonmanual or manual, skilled; IV and V, partly skilled and unskilled. 
7 Includes cohort members who were institutionalized, retired, unemployed, and other.
seasonal subpopulations participating in the third National Health and Nutrition Examination Survey (NHANES III), the average 25(OH)D concentration was 66 nmol/L during winter in the southern group (median latitude 32°N) and 73 nmol/L during the summer in the northern group (median 39°N) (32). The prevalence of 25(OH)D concentrations <25 nmol/L was low (≤3%) in NHANES III (32). The vitamin D status of the general US population is increasingly acknowledged to be unsatisfactory (2, 32, 33), and thus the even higher prevalence of hypovitaminosis D in the British population in the current study than in the US population highlights the urgency of the situation in Great Britain.

The high prevalence of hypovitaminosis D observed in British 45 y-olds is not surprising. First, Great Britain is located between 50°N and 60°N, which corresponds to the latitude of Canada up to the southern tip of Alaska. North of London, no cutaneous vitamin D synthesis occurs in December and January, and, even during the remainder of the year, cloud cover can block up to 99% of vitamin D production (10). The effect of latitude on production was apparent within the current study; the highest rates of hypovitaminosis D were observed in Scottish participants. Second, vitamin D supplements appear to be taken less frequently in Great Britain than in other countries. We found that 13% of men and 20% of women used supplements, whereas the corresponding proportions reported for the United States were 30% and 40% (11). Estimates of supplement use from the current study agree well with those reported from the National Diet and Nutrition Survey (12% and 24%, respectively) (34). Limited availability of supplements (eg, lack of over-the-counter single vitamin D products) is likely to contribute to these differences. In addition, the average dietary intake of vitamin D in the British National Diet and Nutrition Survey was only half of that reported for the United States, which may at least partly reflect differences in food fortification policy (11, 34). Unlike the situation in the United States, milk is not fortified with vitamin D in the United Kingdom, and only fortification of margarine is mandatory. The amount of added vitamin D is relatively low, because the purpose of the fortification is only to increase the vitamin D concentration of margarine to concentrations that occur naturally in butter (25).

In the current study, the frequency of margarine consumption was not associated with the average 25(OH)D concentration, but the risk of 25(OH)D concentrations <25 nmol/L was slightly reduced. This suggests that, whereas fortification of margarine is largely ineffective in improving vitamin D status at population level, it may be sufficient to raise 25(OH)D concentrations in the extreme state of deficiency. However, we acknowledge that only relatively crude information on margarine use was available; hence, we cannot exclude the possibility that underlying measurement error contributed to these observations.

Methodologic considerations

Cutoffs for adequate vitamin D status are a key influence on our perception of the extent hypovitaminosis D. For this reason, we presented data for 3 thresholds that are in part defined by known health outcomes [i.e., <25 nmol/L to reflect calcium malabsorption and rickets (1, 4, 8) and <75 nmol/L from a consensus statement on requirements for bone health (4, 9)]. For an intermediate cutoff (<40 nmol/L), we relied on the view of most laboratories carrying out vitamin D assays that <40 nmol/L was the concentration reflecting the need for vitamin D supplementation, and it is commonly used as the lower reference level by the laboratories (3, 28). An alternative strategy would have been to use a higher threshold of 50 nmol/L (4). Furthermore, 25(OH)D concentrations can vary with assay method (7, 28). We used an automated application that is particularly suitable for large population surveys because of its excellent repeatability and the lack of variation by the operator carrying out the assay (26). Standardization of values to data from DEQAS facilitated the use of previously determined cutoffs for hypovitaminosis D.

The main strength of the study lies in the large sample of whites for whom information on 25(OH)D concentrations was available. With nationwide coverage and measures spanning the year, the current study provides valuable information on the
HYPOVITAMINOSIS D IN GREAT BRITAIN 867

TABLE 3
Adjusted odds ratios (95% CIs) for selected risk factors of hypovitaminosis D (25-hydroxyvitamin D <40 nmol/L) in the 7437 participants of the 1958 British birth cohort

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Simple&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Adjusted&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obesity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Yes</td>
<td>2.03 (1.8, 2.3)</td>
<td>1.83 (1.6, 2.1)</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Region of residence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>South</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Midlands and Wales</td>
<td>1.22 (1.1, 1.4)</td>
<td>1.13 (1.0, 1.3)</td>
</tr>
<tr>
<td>North</td>
<td>1.19 (1.0, 1.4)</td>
<td>1.09 (0.9, 1.3)</td>
</tr>
<tr>
<td>Scotland</td>
<td>2.38 (2.0, 2.9)</td>
<td>2.13 (1.7, 2.6)</td>
</tr>
<tr>
<td>P for trend</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Use of vitamin D supplements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Yes</td>
<td>0.33 (0.28, 0.40)</td>
<td>0.36 (0.30, 0.44)</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Margarine consumption</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than weekly</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Weekly</td>
<td>0.82 (0.69, 0.98)</td>
<td>0.83 (0.69, 0.99)</td>
</tr>
<tr>
<td>Daily</td>
<td>0.92 (0.82, 1.04)</td>
<td>0.87 (0.77, 0.99)</td>
</tr>
<tr>
<td>P for trend</td>
<td>0.23</td>
<td>0.04&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oily fish consumption</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Less than weekly</td>
<td>0.62 (0.53, 0.74)</td>
<td>0.68 (0.57, 0.82)</td>
</tr>
<tr>
<td>Weekly</td>
<td>0.49 (0.41, 0.59)</td>
<td>0.59 (0.48, 0.71)</td>
</tr>
<tr>
<td>P for trend</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Time spent outdoors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(March through November)&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 30 min/d</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>30–59 min/d</td>
<td>0.77 (0.60, 0.98)</td>
<td>0.74 (0.57, 0.95)</td>
</tr>
<tr>
<td>1–2.9 h/d</td>
<td>0.64 (0.51, 0.80)</td>
<td>0.62 (0.49, 0.78)</td>
</tr>
<tr>
<td>3–3.9 h/d</td>
<td>0.56 (0.44, 0.73)</td>
<td>0.48 (0.36, 0.63)</td>
</tr>
<tr>
<td>≥4 h/d</td>
<td>0.47 (0.37, 0.59)</td>
<td>0.37 (0.29, 0.48)</td>
</tr>
<tr>
<td>P for trend</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Use of sun protection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Usually</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Sometimes</td>
<td>0.97 (0.85, 1.11)</td>
<td>0.97 (0.84, 1.12)</td>
</tr>
<tr>
<td>Rarely or never</td>
<td>1.61 (1.31, 1.97)</td>
<td>1.63 (1.32, 2.01)</td>
</tr>
<tr>
<td>P for trend</td>
<td>0.0003</td>
<td>0.0006</td>
</tr>
<tr>
<td>Television viewing or use of computer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 1 h/d</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>1–1.9 h/d</td>
<td>1.00 (0.98, 1.12)</td>
<td>0.92 (0.74, 1.14)</td>
</tr>
<tr>
<td>2–2.9 h/d</td>
<td>1.13 (0.91, 1.40)</td>
<td>0.98 (0.78, 1.24)</td>
</tr>
<tr>
<td>3–3.9 h/d</td>
<td>1.40 (1.12, 1.75)</td>
<td>1.19 (0.95, 1.50)</td>
</tr>
<tr>
<td>≥4 h/d</td>
<td>2.27 (1.80, 2.85)</td>
<td>1.78 (1.40, 2.26)</td>
</tr>
<tr>
<td>P for trend</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<sup>1</sup> Analyses were done by using logistic regression. 25-Hydroxyvitamin D was <40 nmol/L in 2035 participants. P values from log likelihood ratio test or log likelihood trend test as indicated.
<sup>2</sup> Adjusted for sex and month of measurement.
<sup>3</sup> Adjusted for sex, month of measurement, BMI, region, socioeconomic status, skin color, and other variables in the table. Multiple imputation was used to fill in information from participants with unknown values of ≥1 background indicators (n = 1117).
<sup>4</sup> Defined as BMI (in kg/m<sup>2</sup>) ≥30.
<sup>5</sup> The association was not significant after restriction of data to sample with complete data on all background indicators (n = 6520); P = 0.35 (log likelihood ratio trend test).
<sup>6</sup> A significant interaction was found between season and time spent outdoors, P < 0.0001 (log likelihood ratio test). n = 6147; 1420 participants with 25-hydroxyvitamin D <40 nmol/L. No association was found between time spent outdoors and hypovitaminosis D during December through February (P for trend = 0.67).

current prevalence of hypovitaminosis D in British adults. Although the 1958 cohort provides a representative sample of the current adult white population, it is not representative of immigrant ethnic minorities (24). Nevertheless, in agreement with previous reports for ethnic minority groups living in Great Britain (15–17), the prevalence of hypovitaminosis D in 154 non-whites in the cohort was markedly high: 50% had concentrations <25 nmol/L, 80% had concentrations <40 nmol/L, and 100% had concentrations <75 nmol/L during the winter and spring. A further limitation is the self-reported information on dietary vitamin D intake, supplementation, and sun exposure. However, the strong associations observed between the available indicators and serum 25(OH)D concentrations add face validity to our findings, as do the clear seasonal trends.

Use of sun protection is known to reduce vitamin D production in the skin (2). However, in the current study, the use of sun protection was associated with slightly higher (rather than lower) 25(OH)D concentrations. This suggests that the use of sun protection partly reflects levels of sun exposure. The lack of any apparent adverse effects of sun protection on vitamin D status in the current study could indicate that, at the population level, sunscreen and protective clothing are used by those who need them, rather than being seen as an excessively cautious measure strongly interfering with vitamin D synthesis.

Public health implications and conclusions

Data from the 1958 birth cohort suggest that, at different cut-offs for hypovitaminosis D, a substantial public health problem exists in British whites. Obese participants and those living in Scotland were at the highest risk of hypovitaminosis D. However, the prevalence in the general population was very high during the winter and spring, which suggests that, to improve the situation, action is required at a population level rather than at a risk-group level. In the United States, calls have gone out for an increase in vitamin D fortification of foods (11), and the data from the current study suggest that such action is also warranted in the United Kingdom. Vitamin D is currently available without prescription as a dietary supplement only as part of cod liver oil or multivitamin products; hence, a need clearly exists to consider increased availability of over-the-counter supplements. Hypovitaminosis D has been implicated in the development of serious conditions, including diabetes, various types of cancer, and cardiovascular diseases, in addition to its essential role in maintaining bone health (1, 2). The high rates of hypovitaminosis D reported in this study suggest that immediate action is needed to improve the vitamin D status of the British population.

We thank Ian Gibb, Steve Turner, and Marie-Claude Fawcett (Royal Victoria Infirmary, Newcastle-on-Tyne, United Kingdom) for carrying out the 25-hydroxyvitamin D assays, and the Centre for Longitudinal Studies, Institute of Education (original data producers) for providing the data.

EH initiated the substudy of 25-hydroxyvitamin D, carried out statistical analyses, and wrote the manuscript. EH and CP jointly obtained funding for the study, participated in critical evaluation of the findings and in revision of the manuscript, and approved the final version of the manuscript. Neither author had a personal or financial conflict of interest.

REFERENCES
2. Holick MF. Sunlight and vitamin D for bone health and prevention of


Anthropometric measures in middle age after exposure to famine during gestation: evidence from the Dutch famine

Aryeh D Stein, Henry S Kahn, Andrew Rundle, Patricia A Zybert, Karin van der Pal–de Bruin, and LH Lumey

ABSTRACT

Background: Few studies in humans have related maternal undernutrition to the size of the adult offspring.

Objective: The objective was to assess whether reductions in food intake by pregnant women during the Dutch famine of 1944–1945 were related to offspring length, weight, and indexes of adiposity in middle age.

Design: We recruited 1) exposed persons born in western Netherlands between January 1945 and March 1946 whose mothers experienced famine during or immediately preceding pregnancy, 2) unexposed persons born in the same 3 institutions during 1943 or 1947 whose mothers did not experience famine during this pregnancy, and 3) unexposed same-sex siblings of persons in series 1 or 2. Anthropometric measurements \((n = 427\) males and 529 females) were obtained between 2003 and 2005. We defined 4 windows of gestational exposure (by ordinal weeks 1–10, 11–20, 21–30, and 31 through delivery) on the basis of exposure to a ration of \(<900\) kcal/d during the whole 10-wk interval.

Results: Exposure to reduced rations was associated with increased weight and greater indexes of fat deposition at several tissue sites in women but not in men \((P\) for interaction \(<0.01)\). Measures of length and linear proportion were not associated with exposure to famine.

Conclusion: Reduced food availability may lead to increased adiposity later in life in female offspring. Am J Clin Nutr 2007;85:869–76.

KEY WORDS Anthropometric measures, body composition, body mass index, body size, famine, maternal and infant health, Netherlands, nutrition, obesity

INTRODUCTION

Adult body mass is a function of height, girth, and tissue mass and distribution. Each of these measures has independent associations with risk of disease and may have specific associations with early development. Attained height, which is inversely associated with risk of cardiovascular disease (1), is strongly associated with birth length (2). Variations in body proportions, such as the ratio of the leg to trunk lengths, may have their origin in childhood (3) and are independent predictors of the risk of later morbidity and mortality (4). Little is known about the role, if any, of prenatal nutrition in the ontogeny of body proportions.

Birth weight, especially when adjusted for birth length, is positively associated with measures of body size in later life (2). Even so, and despite the consistent association between adult overweight and type 2 diabetes or cardiovascular disease (5), an increased birth weight is also associated with a decreased risk of major chronic diseases (6). An explanation for this apparent paradox might come from information on the sources of variation in size at birth (7), but few studies of humans can document the complex relations extending from maternal nutrition through fetal development and risk of adult disease.

The Dutch famine of 1944–1945 provides a rare opportunity to study the long-term consequences of maternal undernutrition in defined stages of gestation (8, 9). The Dutch famine affected the western Netherlands (10–12). Official rations, which by the end of the famine consisted almost exclusively of bread and potatoes, fell below 900 kcal/d by 26 November 1944 and were as low as 500 kcal/d by April 1945. The famine ceased immediately after liberation. This extraordinary period of deprivation affected fertility, weight gain during pregnancy, maternal blood pressure, and infant size at birth (13–15). The reduction in fertility was greater among manual than among nonmanual occupational classes (8). The decline in mean birth weight of 300 g was restricted to exposure to maternal undernutrition during the third trimester (16, 17).

An earlier investigation of Dutch men aged 19 y found a doubling of the prevalence of overweight with maternal exposure to famine in midgestation (18). A second study, with data collected when the famine-exposed birth cohort was aged 50 y, reported increased body mass index (BMI; in kg/m\(^2\)) in women (but not in men) who were exposed to famine in early gestation (19). To date, no studies have reported on other anthropometric indexes of adiposity after gestation during the Dutch famine. The present study was conducted to replicate the earlier findings, extend follow-up through age 59 y, and analyze a wider array of measures of tissue distribution. We also accounted statistically

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2 The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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for familial determinants of growth and tissue distribution by including same-sex siblings as control subjects.

SUBJECTS AND METHODS

Population source and tracing

We identified 3307 live singleton births (proband) at 3 institutions in famine-exposed cities (midwifery training schools in Amsterdam and Rotterdam and the university hospital in Leiden) in 1945 and early 1946 (100% sample) and in 1943 and 1947 (the first 30 births/no across the 3 institutions). At the time of the famine, a large majority of deliveries (≥70%) in the Netherlands were scheduled to occur at home. The client mix at the 2 midwifery training schools consisted of low-risk pregnancies of women of lower socioeconomic status whose home environment was unsuitable for delivery. The client mix in Leiden included such deliveries as well as women with higher-risk pregnancies identified during prenatal care and emergency admissions after complications of home labor. We extracted personal identifiers, including name and maternal address, birth weight, and other information from the admission logs and delivery progress charts.

To trace the adult offspring, we provided the names and addresses of at birth of all 3307 persons to the population register in the municipality of birth. Of these named persons, 308 (9.3%) were reported to have died and 275 (8.3%) to have migrated. For those who declined, 67% reported not having a same-sex sibling available for study. To increase the overall number of participants, therefore, we attempted to enroll persons who had indicated willingness to participate together with a sibling. Of the 1767 persons, of whom 347 (19.6%) received some reply, 67% reported not having a same-sex sibling available for study. Two measurements were taken for height and weight. Two measurements were taken for other anthropometric outcomes, and the mean was used for the analysis. To ensure independence of the replicate measures, all markings of measurement points were erased before the second measurement was obtained. If the first 2 measurements were not sufficiently close (arm length, waist circumference, mid thigh circumference 1.0 cm; subscapular skinfold thicknesses were obtained to the nearest 0.2 mm with calipers with a maximal spread of 40 mm (Holtain)). These calipers were calibrated daily. A single measurement was taken for height and weight. Two measurements were taken for other anthropometric outcomes, and the mean was used for the analysis. To ensure independence of the replicate measures, all markings of measurement points were erased before the second measurement was obtained. If the first 2 measurements were not sufficiently close (arm length, waist circumference, mid thigh circumference 1.0 cm; subscapular skinfold thicknesses were obtained to the nearest 0.2 mm with calipers with a maximal spread of 40 mm (Holtain)).

Recruitment and examination

Traced persons were mailed a letter of invitation signed by the current director of the institution in which they were born, a brochure describing the study, and a response card. We mailed one reminder letter to nonresponders. Initially, our study design called for the recruitment of same-sex sibling pairs; hence, the lack of an available sibling was a reason for ineligibility. We received some reply from 1767 persons, of whom 347 (19.6%) expressed willingness to participate together with a sibling. Of those who declined, 67% reported not having a same-sex sibling available for study. To increase the overall number of participants, therefore, we attempted to enroll persons who had indicated ineligibility because of the lack of an available sibling.

We conducted a telephone interview, which was followed by a clinical examination at the Leiden University Medical Center. Most of the clinical examinations were conducted within 6 wk of the telephone interview. All study protocols were approved by the human subjects committees of the participating institutions, and participants provided verbal consent at the start of the telephone interview and written informed consent at the start of the clinical examination. We obtained anthropometric measurements from 971 subjects (437 men and 534 women): 311 proband-sibling pairs, 2 siblings whose matching proband did not complete the clinical examination, and 347 additional probands.

Anthropometric measures

All anthropometric measures were obtained by experienced research nurses, who were provided specific training in the methods by one of us (HSK); only trivial differences in means or in the variability of measures across nurses were observed. Weight was obtained to the nearest 100 g with the participant standing on a portable digital scale (SECA, Hamburg, Germany). Standing height was measured to the nearest 1 mm with a portable stadiometer (SECA), and seated height was obtained to the nearest 1 mm with the participant seated on a hard stool of known height with the use of the same stadiometer. Right arm length (tip of acromion to the distal tip of the third metacarpal bone) and waist (at level of iliac crests, intersection with midaxillary line), hip (buttocks at the point of maximum extension), and right mid thigh (supine with hip flexed at 45°, between lateral inguinal crease and proximal patella) circumferences were obtained to the nearest 1 mm with the use of a nonextensible measuring tape (Hoechstmass, Sulzbach, Germany). The supine sagittal abdominal diameter (SAD) at the level of the iliac crests was obtained to the nearest 1 mm with a sliding-beam caliper (Holtain, Dyfed, Wales, United Kingdom). Tricipital, subscapular, and anterior mid thigh skinfold thicknesses were obtained to the nearest 0.2 mm with calipers with a maximal spread of 40 mm (Holtain). These calipers were calibrated daily. A single measurement was taken for height and weight. Two measurements were taken for other anthropometric outcomes, and the mean was used for the analysis. To ensure independence of the replicate measures, all markings of measurement points were erased before the second measure was obtained. If the first 2 measurements were not sufficiently close (arm length, waist circumference, mid thigh circumference 1.0 cm; subscapular diameter 0.5 cm; subscapular or triceps skinfold thickness 2.0 mm) a third and fourth measure were taken and the 3 measures closest together from the 4 available measures were averaged.

Derived measures

Trunk length was calculated by subtracting the height of the stool from seated height, and leg length was obtained by subtracting trunk length from standing height. As indexes of body proportion, we computed the ratios of the right arm to leg lengths and the leg to trunk lengths. We computed the BMI. As additional indexes of mass distribution, we computed the ratios of waist to hip circumference, waist to mid thigh circumference, and SAD to mid thigh circumference. We excluded from the analysis individuals for whom any of the above anthropometric measures were missing (n = 12), for whom the ratio of trunk to leg length exceeded 1.10 (n = 2), and one man with polio-related atrophy of a lower limb; the analytic sample consisted of 956 subjects.

Because several of the participants had one or more skinfold thicknesses that exceeded the capacity of the calipers, we categorized the skinfold thicknesses into empirical quartiles. We then developed a 3-level indicator of the relative distribution of subcutaneous fat between the triceps and subscapular regions by cross-tabulating the quartile distributions for these 2 regions. We coded this indicator as −1 if the triceps value was in a higher quartile of the distribution than was the subscapular value, as +1 if the reverse was true, and 0 if both were in the same quartile.

Categorizing exposure to famine

We defined the start of each gestation by the date of the mother’s last menstrual period (LMP), as noted in the original prenatal record, unless it was missing or the resulting gestational age was implausible (12.4%). In these cases, we approximated the date of LMP from the unambiguous date of birth and estimates of gestational age recorded on the birth record or from a gestational age...
estimate based on sex- and parity-specific birth weights of singleton live births at the Amsterdam midwives school and the University of Amsterdam Department of Obstetrics between 1931 and 1965 at each gestation between 24 and 46 wk (20).

We characterized exposure to famine during gestation by determining the weeks after the LMP during which the mother was exposed to an official ration of <900 kcal/d (the 24 wk included the period from 26 November 1944 to 12 May 1945). We defined the mother as exposed in specific periods if gestational weeks 1–10, 11–20, 21–30, or 31 to delivery were entirely included in this time window. Thus, pregnancies with an LMP between 26 November 1944 and 4 March 1945 (n = 74) were considered exposed in weeks 1–10, between 18 September 1944 and 24 December 1944 (n = 124) exposed in weeks 11–20, between 10 July 1944 and 15 October 1944 (n = 140) exposed between weeks 21–30, and between 2 May 1944 and 24 August 1944 (n = 128) exposed between week 31 and delivery. Because these time windows overlap, the participants could be considered exposed during one or (at most) two 10-wk periods; those exposed in at least one 10-week period (n = 350) were considered to have some exposure to famine. In this formulation, the estimate for the variable “any exposure to famine” is not necessarily an average of the estimates for the four 10-wk periods, because these may have independent and additive or counteractive associations with adult size.

Statistical methods

We computed means and SDs or categorical distributions, as appropriate. We developed independent linear regression models for all models that did not include skinfold thicknesses. Skinfold thicknesses and their ratios were analyzed by using logistic regression, with the highest category compared against all others. Because humans are sexually dimorphic and previous research has identified associations of famine with body composition in one or the other sex (18, 19), we tested for heterogeneity of associations by sex using F tests. We considered a P value <0.10 to indicate an interaction and conducted sex-stratified analyses where indicated.

We considered a set of models and regressed each outcome variable separately on exposure to famine. Exposure to famine was characterized by using an indicator for any gestational exposure, with the reference category being no exposure, and by using the four 10-wk intervals described, which we entered as a set of 4 indicator variables. We used the combined population of control subjects (unexposed births in the 3 hospitals: n = 296; siblings of the birth series: n = 310) as the reference, and we adjusted for age at examination. We used the xtreg and xlogit commands in STATA 8 (Stata Corp, College Station, TX) to control for clustering within families. We assessed whether associations with exposure to famine were mediated through birth weight or length by entering these terms and comparing the changes in coefficients. These analyses were run on the institutional birth series alone (birth weight: n = 297 men and 348 women; birth length: n = 278 men and 325 women; we lacked information on size at birth for the sibling control subjects). Estimates of the effect of exposure to famine were similar in models that included the birth series and those that included the birth series and the siblings.

We examined whether measurement error in seated height because of excess adiposity in the buttocks might affect relations by adding hip circumference to the relevant models. In practice, this adjustment had no effect on any observed associations (data not shown), and models without this adjustment are presented. Models for circumferences, the SAD, and their resulting ratio measures included a term for standing height to account for allometric scaling; we also tested whether these associations were affected by body proportion by including a term for the leg-to-trunk ratio. Although the outcome measures were generally associated with both height and the leg-to-trunk ratio, addition of these terms did not alter the observed associations between the measures and exposure to famine (data not shown), and we present results without this adjustment. Models for indexes of mass distribution included adjustment terms describing adult measures that might be causally related to adiposity, including smoking status, intake of alcohol, intake of energy as estimated from a food-frequency questionnaire, physical activity level in the year before the examination as assessed by the SQUASH questionnaire (21), and, for women, parity. In practice, control for these factors did not affect the estimates (data not shown). We did not consider these variables relevant for analyses of lengths and body proportion because these outcomes are established by early adulthood.

RESULTS

Differences between traced and untraced persons

The proportion of participants identified as deceased was highest among probands born in 1943 (10.4%) and lowest among probands born in 1947 (6.0%). Status as an emigrant or other reasons why a current address was not found did not differ by year of birth or period of exposure to famine. When we compared the birth records of participants traced to a current address with those who had either died, emigrated, or had not been located we found no clinically significant differences in mean birth weight (3350 compared with 3314 g) or length (50.4 compared with 50.2 cm), placental weight (601 compared with 592 g), maternal age at delivery (28.2 compared with 27.4 y), or birth order (2.3 compared with 2.3).

Differences between interviewed and noninterviewed persons

Of the 2300 persons who were invited to join the study, we found no significant differences between those interviewed to those who were not interviewed in mean birth weight (3374 compared with 3339 g) or length (50.5 compared with 50.3 cm), placental weight (600 compared with 601 g), maternal age at delivery (28.6 compared with 28.1 y), or birth order (2.4 compared with 2.2). The response to our invitation, however, was lower for those born in 1947 (25%) than in all others (35%). Eleven percent of those who were interviewed lived within 5 km of the examination site compared with 10% of those who were not interviewed, and 34% of those interviewed lived >45 km from the examination site compared with 29% of those who were not interviewed.

Final sample for analysis

We analyzed anthropometric data (except for skinfold thicknesses) from 956 persons. On the basis of their behavior and anthropometric measures, these persons appeared unremarkable for Dutch populations of this age (Table 1 and Table 2).
Exposure to famine and measures of length and body proportions

There was no evidence of a statistical interaction by sex in the association of maternal exposure to famine with measures of offspring length or their ratios (data not shown). There was no overall association between exposure to famine and these measures when famine was considered as a whole (Table 3); when considered as 4 periods of gestation, the ratio of the arm to leg lengths showed gestation-period—specific associations, which increased \( P < 0.10 \) after exposure in weeks 21–30 and decreased \( P < 0.05 \) after exposure in weeks 31 through delivery.

Exposure to famine and indexes of mass and mass distribution

Strong statistical evidence for interaction by sex in the association of any famine exposure was found for all indexes of mass distribution \( P \) for heterogeneity < 0.001) except waist-to-hip ratio. For men, no association between any exposure to famine and any index was found, whether considered individually or when the 4 periods were considered as a group \( (P > 0.10 \) for all). In contrast, all the indexes were elevated in women exposed to famine \( (P < 0.05 \) for all, except the waist-to-hip ratio, for which \( P < 0.10 \) \( (P < 0.01 \)) for all measures, except the ratios of the arm-to-hip circumferences and waist to mid thigh circumferences \( P > 0.05 \) for both). Inspection of the period-specific estimates suggested similarities between men and women for the estimates for exposure in gestational weeks 1–10 and substantial divergence between men and women in the estimates for exposures in later 10-wk periods.

Skinfold thicknesses

In sex-pooled analyses (Table 6), the odds of being in the highest quartile of the subscapular skinfold thickness and the ratio of the subscapular to tricipital skinfold thickness were modestly elevated with any exposure to famine \( P < 0.10 \). The test for interaction by sex was not significant \( (P > 0.10 \) in age-adjusted models) for any skinfold thickness. There was no strong indication of association with specific periods of exposure to famine.

Analyses on birth series alone

We repeated all analyses using the 645 participants with measures of birth weight and the 603 participants with measures of birth length. Results were very consistent with those reported for the whole sample (data not shown). In these groups, the results did not change when birth weight or birth length were included in the model (data not shown).

DISCUSSION

In a follow-up study of persons exposed during gestation to the Dutch famine of 1944–1945, we observed that maternal exposure to acute famine is associated with increases in several indexes of body mass and mass distribution among female offspring at age 59 y. We did not observe any strong independent association of prenatal exposure to famine with adult lengths or body proportions.

The circumstances of the Dutch famine provide a model to test for isolated effects of undernutrition at defined stages of development and do not speak to the situation in which inadequate prenatal nutrition is followed by continued undernutrition, as...
was until recently common in many developing countries. Exposure to famine, as we defined it in relation to official rations, is an ecologic measure of undernutrition; we lacked individual dietary intake data. However, evidence of the severity of the famine was abundant, including evidence that during the height of the famine pregnant women actually lost weight over the second half of their pregnancy (15). Thus, our data support the notion that maternal undernutrition in gestation, if postnatal nutrition and infections are not limiting, neither programs a person for an altered trajectory of linear growth if it occurs in early pregnancy nor results in unrecoverable deficits in attained length if it occurs later in gestation. In women, however, the prenatal deprivation appears to have been associated with increased weight in middle age, with more of the increased mass deposited centrally.

Two earlier studies of persons exposed to the Dutch famine in utero have yielded mixed results. Among men examined at age 18 y, the absolute risk of obesity (defined as >120% of the ideal weight for height according to the Metropolitan Life Insurance Company tables) was elevated from 1.5% to 2.8% with exposure in midgestation (18). Our study lacked the power to detect an effect of that small a magnitude. A study similar in design to ours found an elevated BMI in women aged 50 y whose mothers were exposed to the famine early in gestation, but there was no association with other periods of exposure to famine or among men (19). Our results are broadly consistent with that study insofar as we also observed a marked difference in associations between men and women, but we did not identify early gestation as being the critical window for effects in adulthood. A third study of the consequences of exposure to famine, conducted among survivors of the siege of Leningrad, did not suggest any difference in BMI between those born before the siege commenced, born during the siege, or born in an area not subject to the siege (22). That study was unable to assess the timing of exposure to maternal undernutrition because the Leningrad siege lasted >2 y. All of the earlier studies considered only weight and height; we examined a wider range of anthropometric dimensions and indexes. We observed some suggestion that the heterogeneity of associations between famine exposure and adult body mass and mass distribution between men and women is established only after the first 10-wk period of gestation. This may reflect the increasing importance of sex-specific growth factors in fetal development (23).

There is ongoing debate about the relative utility of the available indexes of body mass distribution in predicting risk for chronic disease (24-26). Although BMI is widely used, it does

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Selected body measurements and ratios for Dutch men and women examined between 2003 and 2005, by famine exposure and sex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exposed to famine during gestation (n = 160)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>177.4 ± 6.2</td>
</tr>
<tr>
<td>Trunk length (cm)</td>
<td>92.6 ± 3.2</td>
</tr>
<tr>
<td>Leg length (cm)</td>
<td>84.7 ± 4.3</td>
</tr>
<tr>
<td>Arm length (cm)</td>
<td>66.8 ± 3.3</td>
</tr>
<tr>
<td>Ratio of arm to leg lengths (× 100)</td>
<td>78.9 ± 3.1</td>
</tr>
<tr>
<td>Ratio of leg to trunk lengths (× 100)</td>
<td>91.5 ± 4.5</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>87.6 ± 12.1</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>100.5 ± 10.1</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>102.7 ± 6.6</td>
</tr>
<tr>
<td>Supine sagittal abdominal diameter (cm)</td>
<td>23.8 ± 3.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.8 ± 3.6</td>
</tr>
<tr>
<td>Midcalf circumference (cm)</td>
<td>52.1 ± 3.8</td>
</tr>
<tr>
<td>Ratio of waist to hip circumferences (× 100)</td>
<td>97.7 ± 5.5</td>
</tr>
<tr>
<td>Ratio of supine sagittal abdominal diameter to midcalf circumference (× 100)</td>
<td>45.6 ± 4.8</td>
</tr>
<tr>
<td>Ratio of waist to midcalf circumferences (× 10)</td>
<td>19.3 ± 1.5</td>
</tr>
<tr>
<td>Subscapular skinfold thickness (mm)</td>
<td>21.0 ± 8.3 [151]</td>
</tr>
<tr>
<td>Triceps skinfold thickness (mm)</td>
<td>12.9 ± 4.3</td>
</tr>
<tr>
<td>Anterior midcalf skinfold thickness (mm)</td>
<td>14.2 ± 7.5 [145]</td>
</tr>
</tbody>
</table>

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1. Born in the same institution and not exposed to famine during gestation.
2. ± SD (all such values).
3. Sample sizes for skinfold thicknesses include subjects in whom skinfold thicknesses were measured but for whom the skinfold thickness exceeded the caliper capacity.
4. All values are medians and interquartile intervals; n in brackets.
5. The 75th percentile for this group exceeded the maximum caliper capacity of 40 mm. The 25th percentile was 23.9 mm.
956 persons examined between 2003 and 2005

Association of exposure to the Dutch famine overall or in the specified period of gestation with weight, circumferences, and indicators of body composition

TABLE 3

<table>
<thead>
<tr>
<th>Period of gestational exposure</th>
<th>Overall (n = 350)</th>
<th>Weeks 1–10 (n = 74)</th>
<th>Weeks 11–20 (n = 124)</th>
<th>Weeks 21–30 (n = 140)</th>
<th>Week 31 to delivery (n = 128)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>−0.30, 1.11</td>
<td>−0.30, 1.72, 1.13</td>
<td>−0.35, 1.51, 0.82</td>
<td>−1.01, 2.13, 0.11</td>
<td>0.51, 0.62, 1.63</td>
</tr>
<tr>
<td>Trunk (cm)</td>
<td>0.11, 0.62</td>
<td>0.11, 0.73, 0.49</td>
<td>−0.12, −0.87, 0.30</td>
<td>0.17, −0.41, 0.76</td>
<td></td>
</tr>
<tr>
<td>Leg (cm)</td>
<td>−0.40, 0.90</td>
<td>−0.47, 1.45, 0.52</td>
<td>−0.26, 1.07, 0.55</td>
<td>−0.72, 1.50, 0.06</td>
<td>0.29, −0.49, 1.07</td>
</tr>
<tr>
<td>Arm (cm)</td>
<td>−0.23, −0.65</td>
<td>−0.41, −1.21, 0.40</td>
<td>−0.13, −0.80, 0.54</td>
<td>−0.07, −0.71, 0.57</td>
<td>−0.13, −0.77, 0.51</td>
</tr>
<tr>
<td>Indexes of proportion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio of arm to leg lengths</td>
<td>0.10, 0.28</td>
<td>0.07, −0.65, 0.79</td>
<td>0.26, −0.34, 0.87</td>
<td>0.58, 0.00, 1.16</td>
<td></td>
</tr>
<tr>
<td>Ratio of leg to trunk lengths</td>
<td>−0.44, 1.00</td>
<td>−0.75, −1.83, 0.34</td>
<td>−0.26, −1.16, 0.64</td>
<td>−0.53, −1.39, 0.34</td>
<td>0.30, −0.56, 1.17</td>
</tr>
</tbody>
</table>

1 Values represent differences from control group (n = 606). Estimates were obtained by linear regression and were adjusted for sex, age, and clustering of siblings. Models for each specific 10-wk period of gestational exposure were also adjusted for exposure in overlapping 10-wk periods. Estimates for any exposure may reflect additive effects of exposure in specific periods. Tests for interaction by sex were not significant (P > 0.25 for each outcome).

2 Values reflect the overall test of association of all 4 periods of exposure considered as a group (Wald test, 4 df).

not differentiate between lean and fat tissue. The ratio of the subscapular and tricipital skinfold thicknesses, a widely used index of the distribution of subcutaneous fat, rather than of increased visceral fat, was only weakly associated in the present study with exposure to famine. Similarly, the waist-to-hip ratio, a presumed correlate of ischemic heart disease (27), was modestly associated with exposure in our study. We found, however, that exposure to the famine was associated among women with an increased ratio of SAD to the midthigh circumference—an alternative anthropometric correlate of ischemic heart disease (28). To date, there have been suggestions that exposure to famine in specific periods of gestation is associated with impaired glucose tolerance (29) and with prevalent coronary heart disease (30), inconsistently associated with blood pressure (31, 32), and

TABLE 4

<table>
<thead>
<tr>
<th>Period of gestational exposure</th>
<th>Overall (n = 160)</th>
<th>Weeks 1–10 (n = 35)</th>
<th>Weeks 11–20 (n = 59)</th>
<th>Weeks 21–30 (n = 69)</th>
<th>Week 31 to delivery (n = 59)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight and circumferences</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>0.98, 1.22</td>
<td>3.37, −0.73, 7.46</td>
<td>−1.63, −5.19, 1.93</td>
<td>2.08, −1.31, 5.47</td>
<td>−1.17, −4.49, 2.16</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>0.51, 1.40</td>
<td>1.82, −1.73, 5.37</td>
<td>−1.28, −4.37, 1.81</td>
<td>1.88, −1.06, 4.82</td>
<td>−0.37, −3.25, 2.51</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>0.73, −0.43</td>
<td>1.94, −2.07, 4.15</td>
<td>−0.48, −2.38, 1.43</td>
<td>0.93, −0.89, 2.76</td>
<td>−0.43, −2.21, 1.36</td>
</tr>
<tr>
<td>Supine sagittal abdominal diameter (cm)</td>
<td>0.20, −0.38</td>
<td>0.84, −0.26, 1.93</td>
<td>−0.42, −1.37, 0.54</td>
<td>0.37, −0.54, 1.28</td>
<td>0.01, −0.88, 0.90</td>
</tr>
<tr>
<td>Midthigh circumference (cm)</td>
<td>0.11, −0.64</td>
<td>1.04, −0.34, 2.42</td>
<td>0.22, −0.99, 1.43</td>
<td>−0.07, −1.22, 1.07</td>
<td>0.73, −1.85, 0.40</td>
</tr>
<tr>
<td>Indexes of mass distribution</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.32, −0.37</td>
<td>1.06, −0.23, 2.34</td>
<td>−0.49, −1.61, 0.63</td>
<td>0.66, −0.41, 1.72</td>
<td></td>
</tr>
<tr>
<td>Ratio of waist to hip</td>
<td>−0.27, 1.34</td>
<td>0.01, −1.97, 1.98</td>
<td>0.86, −2.59, 0.86</td>
<td>0.82, −0.82, 2.46</td>
<td></td>
</tr>
<tr>
<td>circumferences (× 100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.11, −1.50, 1.72</td>
</tr>
<tr>
<td>Ratio of supine sagittal</td>
<td>0.31, −0.61</td>
<td>0.70, −1.00, 2.40</td>
<td>−0.86, −2.34, 0.62</td>
<td>0.66, −0.75, 2.06</td>
<td></td>
</tr>
<tr>
<td>abdominal diameter to</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.76, −0.62, 2.14</td>
</tr>
<tr>
<td>midthigh circumference (× 100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio of waist to midthigh</td>
<td>0.50, −2.47</td>
<td>−0.17, −5.63, 5.29</td>
<td>−2.91, −7.68, 1.86</td>
<td>3.43, −1.10, 7.96</td>
<td>2.20, −2.25, 6.64</td>
</tr>
</tbody>
</table>

1 Values represent differences from control group (n = 267). Estimates were obtained by linear regression and were adjusted for age, height, and clustering of siblings. Estimates for specific 10-wk periods of gestational exposure were also adjusted for exposure in overlapping 10-wk periods. Estimates for any exposure may reflect the additive effects of exposure in specific periods.

2 Values reflect the overall test of association of all 4 periods of exposure considered as a group (Wald test, 4 df).
TABLE 5
Association of exposure to the Dutch famine overall or in the specified period of gestation with weight, circumferences, and indexes of adiposity in adulthood for 529 women measured between 2003 and 2005

<table>
<thead>
<tr>
<th>Period of gestational exposure</th>
<th>Overall (n = 190)</th>
<th>Weeks 1–10 (n = 59)</th>
<th>Weeks 11–20 (n = 65)</th>
<th>Weeks 21–30 (n = 71)</th>
<th>Week 31 to delivery (n = 69)</th>
<th>$P^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight and circumferences</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>Estimate 95% CI</td>
<td>Estimate 95% CI</td>
<td>Estimate 95% CI</td>
<td>Estimate 95% CI</td>
<td>Estimate 95% CI</td>
<td></td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>4.83 1</td>
<td>2.51, 7.14</td>
<td>3.98, −0.53, 8.48</td>
<td>3.71, 0.04, 7.39</td>
<td>3.53, 0.02, 7.05</td>
<td>2.75, −0.77, 6.26 &lt;0.01</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>4.69 1</td>
<td>2.66, 6.72</td>
<td>1.96, −1.93, 5.85</td>
<td>2.83, 0.64, 7.02</td>
<td>3.88, 0.83, 6.93</td>
<td>2.50, −0.53, 5.53 &lt;0.01</td>
</tr>
<tr>
<td>Supine sagittal abdominal diameter (cm)</td>
<td>1.52 1</td>
<td>0.91, 2.13</td>
<td>1.00, −0.18, 2.18</td>
<td>1.13, 0.17, 2.10</td>
<td>0.84, −0.08, 1.77</td>
<td>1.34, 0.42, 2.26 &lt;0.01</td>
</tr>
<tr>
<td>Midthigh circumference (cm)</td>
<td>1.61 1</td>
<td>0.65, 2.57</td>
<td>1.01, −0.86, 2.88</td>
<td>1.40, −0.12, 2.92</td>
<td>1.64, 0.18, 3.09</td>
<td>0.56, −0.90, 2.01 &lt;0.01</td>
</tr>
<tr>
<td>Indexes of mass distribution</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>1.85 2</td>
<td>1.01, 2.69</td>
<td>1.44, −0.21, 3.09</td>
<td>1.45, 0.11, 2.80</td>
<td>1.34, 0.06, 2.62</td>
<td>1.08, −0.20, 2.37 &lt;0.01</td>
</tr>
<tr>
<td>Ratio of waist to hip circumferences ($\times$ 100)</td>
<td>0.89 2</td>
<td>−0.15, 1.83</td>
<td>0.09, −1.80, 1.99</td>
<td>0.33, −1.22, 1.88</td>
<td>1.09, −0.04, 2.57</td>
<td>0.17, −1.31, 1.64 NS</td>
</tr>
<tr>
<td>Ratio of supine sagittal abdominal diameter to midthigh circumference ($\times$ 100)</td>
<td>1.54 2</td>
<td>0.69, 2.39</td>
<td>1.28, −0.35, 2.91</td>
<td>0.90, −0.44, 2.23</td>
<td>0.33, −0.94, 1.61</td>
<td>2.09, 0.82, 3.36 &lt;0.01</td>
</tr>
<tr>
<td>Ratio of waist to midthigh circumferences ($\times$ 100)</td>
<td>3.34 2</td>
<td>0.38, 6.29</td>
<td>1.84, −3.82, 7.50</td>
<td>1.79, −2.85, 6.44</td>
<td>2.28, −2.16, 6.72</td>
<td>2.71, −1.70, 7.13 NS</td>
</tr>
</tbody>
</table>

1 Values represent differences from control group (n = 339). Estimates were obtained by linear regression and were adjusted for age, height, and clustering of siblings. Estimates for specific 10-wk periods of gestational exposure were also adjusted for exposure in overlapping 10-wk periods. Estimates for any exposure may reflect the additive effects of exposure in specific periods.

2 Odds ratios are for the highest quartile compared with all others.

3 $P < 0.01$.

4 $P < 0.10$.

5 $P < 0.05$.

not associated with overall mortality (33); all these conditions have shown associations with adiposity. Thus, future research needs to consider how differences in adiposity consequent to exposure to famine during gestation, including differences in the distribution of lean and adipose tissue throughout the body, might mediate any effect of the famine on risk of disease.

It is possible that participation bias may have led to our findings if heavy women with famine exposure were more likely to

TABLE 6
Association of exposure to the Dutch famine overall or in the specified period of gestation with selected skinfold thicknesses in adulthood for persons measured between 2003 and 2005

<table>
<thead>
<tr>
<th>Period of gestational exposure</th>
<th>Overall</th>
<th>Weeks 1–10</th>
<th>Weeks 11–20</th>
<th>Weeks 21–30</th>
<th>Week 31 to delivery</th>
<th>$P^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subscapular ($n = 929$)</td>
<td>1.38 4</td>
<td>0.95, 1.99</td>
<td>0.87, 0.43, 1.76</td>
<td>1.35, 0.77, 2.37</td>
<td>1.09, 0.64, 1.88</td>
<td>1.42, 0.82, 2.44 NS</td>
</tr>
<tr>
<td>Tricipital ($n = 935$)</td>
<td>1.30 4</td>
<td>0.89, 1.89</td>
<td>1.50, 0.75, 3.00</td>
<td>1.16, 0.64, 2.07</td>
<td>1.09, 0.63, 1.91</td>
<td>1.37, 0.79, 2.38 NS</td>
</tr>
<tr>
<td>Anterior midthigh ($n = 760$)</td>
<td>1.21 4</td>
<td>0.80, 1.81</td>
<td>1.18, 0.57, 2.47</td>
<td>1.10, 0.59, 2.06</td>
<td>1.35, 0.73, 2.52</td>
<td>0.84, 0.45, 1.58 NS</td>
</tr>
<tr>
<td>Ratio of subscapular to tricipital ($n = 916$)</td>
<td>1.55 4</td>
<td>1.08, 2.22</td>
<td>1.07, 0.55, 2.08</td>
<td>1.67, 0.96, 2.92</td>
<td>1.46, 0.86, 2.49</td>
<td>1.09, 0.63, 1.87 &lt;0.10</td>
</tr>
</tbody>
</table>

1 Estimates were obtained by logistic regression and were adjusted for sex, age, height, and clustering of siblings. Tests for interaction by sex were not significant ($P > 0.10$). Estimates for specific 10-wk periods of gestational exposure were also adjusted for exposure in overlapping 10-wk periods.

2 Odds ratios are for the highest quartile compared with all others.

3 $P < 0.10$.

4 Odds ratios are for the group in which the subscapular skinfold is in a higher quartile than is the tricipital skinfold compared with all others.

5 $P < 0.05$. 
participate in our study than were heavy women with no famine exposure. We have no method to test for this potential bias, however. We note that participation rates did not differ by sex or by distance from the examination site. It is also possible that parental characteristics associated with offspring adiposity differed by period of maternal exposure to famine. The effect of such bias was minimized in our study because we selected control subjects from among siblings born outside of the famine period (thus controlling for genetic sources of variation in adult adiposity) and among births in the same institutions (thus minimizing social class differences between exposed and unexposed persons). Adjustment for several variables that are themselves predictors of adiposity, including measures of energy balance and, in women, parity, did not affect our measures of association between famine exposure and body mass distribution of the offspring.

In conclusion, exposure to the Dutch famine was strongly associated with a wide range of indexes of body mass distribution in middle-age women, and it was not associated with these indexes in men or with measures of length or body proportions in either men or women. These data suggest sex-specific, long-lasting effects of maternal undernutrition during pregnancy.

We thank the Vroedvrouwen scholen of Amsterdam and Rotterdam and the Obstetrics Department of the Leiden University Medical Center for their help in accessing their archives. The clinical examinations were carried out at the study center of Gerontology & Geriatrics, Leiden University Medical Center, under supervision of L. de Man (head of study center).

LHL, ADS, and HSK developed the study hypothesis and study protocols, designed the study, and developed and coordinated all data collection activities. LHL obtained the major funding. ADS conducted the data analysis and wrote the initial drafts of the manuscript. LHL and HSK participated in the data interpretation. KvDP participated in the development of the data collection protocols and initial data management and in data interpretation. PAZ managed the files and data cleaning and participated in the data interpretation. AR participated in the data analysis and interpretation. All authors reviewed and approved the final version of the manuscript. None of the authors declared any financial conflict of interest.

REFERENCES

Prospective study of intake of fruit, vegetables, and carotenoids and the risk of adult glioma\textsuperscript{1–3}

Crystal N Holick, Edward L Giovannucci, Bernard Rosner, Meir J Stampfer, and Dominique S Michaud

ABSTRACT

Background: Nutrients in dietary fruit and vegetables have been hypothesized to lower the risk of glioma by reducing the endogenous formation of N-nitroso compounds. Studies examining fruit and vegetable consumption and brain tumors have relied on case-control study designs, with one exception, and results have been inconsistent.

Objective: We prospectively examined the relation between consumption of fruit and vegetables (and specifically carotenoids) and the risk of glioma among men and women in 3 large US cohort studies: the Health Professionals Follow-Up Study (HPFS), the Nurses’ Health Study I (NHS I), and NHS II.

Design: Dietary intake was assessed by food-frequency questionnaires obtained at baseline and updated every 4 y through 2002 (HPFS and NHS I) or 2003 (NHS II). We identified 296 incident adult gliomas during 3 669 589 person-years of follow-up. Cox proportional hazard models were used to estimate incidence rate ratios (RR) and 95% CIs between intake of fruit, vegetables, and carotenoids and glioma risk, with adjustment for age and total caloric intake.

Results: Updated average consumption of total fruit and vegetables was not significantly associated with glioma risk in the men and women (pooled multivariate RR in a comparison of the highest with the lowest quintile: 1.12; 95% CI: 0.74, 1.69). Other fruit and vegetable subgroups, individual fruit and vegetables, and 5 major carotenoids were not significantly associated with glioma in the men and women (pooled multivariate RR in a comparison of the highest with the lowest quintile: 1.12; 95% CI: 0.74, 1.69). Other fruit and vegetable subgroups, individual fruit and vegetables, and 5 major carotenoids were not significantly associated with glioma.

Conclusion: Our findings suggest that fruit, vegetable, and carotenoid consumption is not likely associated strongly with the risk of adult glioma.


KEY WORDS Fruit, vegetables, glioma, prospective studies, epidemiology

INTRODUCTION

Age-adjusted incidence rates for primary malignant brain tumors range from 6.0 to 8.7 per 100 000 person-years across 16 of the US states participating in the Central Brain Tumor Registry of the United States (CBTRUS) \textsuperscript{(1)}. Although brain tumors are uncommon, they are associated with significant mortality and morbidity; the estimated 5-y survival rates are \textasciitilde30% for men and women \textsuperscript{(1)}. Gliomas represent the most common type of adult brain tumor \textasciitilde(77% of malignant brain tumors) \textsuperscript{(1)}.

Established risk factors for glioma include increasing age, male sex, white race, and inherited factors (eg, Li-Fraumeni syndrome). Studies that have examined the association between fruit and vegetable intake and brain tumors have relied almost entirely on case-control study designs, with one exception \textsuperscript{(2)}, and results have been inconsistent \textsuperscript{(2–14)}. Limitations include the small number of glioma cases \textsuperscript{(2, 5, 8, 10)}, potential recall or selection bias \textsuperscript{(3, 5, 8–10, 12)}, and measurement error due to surrogates of information \textsuperscript{(3, 4, 6–8, 11, 13, 14)}. Nutrients and phytochemicals in fruit and vegetables have been hypothesized to lower the risk of glioma by reducing the endogenous formation of N-nitroso compounds (NOCs), which have been associated with elevated glioma risk \textsuperscript{(15)}. Vitamins C and E and phenolics have been shown to block endogenous nitrosation of nitrites \textsuperscript{(16–18)}. These dietary constituents and carotenoids may act as potent antioxidants and inhibit free radical generation and oxidative stress; through these mechanisms, fruit and vegetable consumption may reduce cancer risk, including the risk of gliomas \textsuperscript{(19–21)}. Given the limitations of previous studies that evaluated the role of fruit and vegetable intake and the risk of glioma, we conducted a prospective investigation in 3 large prospective studies of men and women with up to 22 y of follow-up, which represents the largest prospective study of this type.

SUBJECTS AND METHODS

Study populations

The Nurses’ Health Study I (NHS I) was initiated in 1976, when 121 700 registered US female nurses aged 30–55 y returned a mailed questionnaire that assessed information on lifestyle factors and medical and smoking histories. Similarly, the Health Professionals Follow-Up Study (HPFS) is a cohort of 51 529 US male physicians, dentists, optometrists, osteopaths, podiatrists, pharmacists, and veterinarians who were 40–75 y of age at enrollment in 1986. The study design and methods of dietary assessment and follow-up for the Nurses’ Health Study II (NHS II) are very similar to those of NHS I. In 1989, 116 686

\textsuperscript{1} From the Departments of Nutrition (CNH, ELG, and MJS), Epidemiology (ELG, MJS, and DSM), and Biostatistics (BR), Harvard School of Public Health, Boston, MA, and the Channing Laboratory, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA (ELG, BR, MJS, and DSM).

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women aged 25–42 y and living in 14 US states were enrolled into the NHS II. Follow-up questionnaires are mailed biennially to all cohort members to update information on lifestyle factors and newly diagnosed medical conditions. The follow-up rate for the cohorts for incidence of cancer was >95% of the total possible person-years.

**Dietary assessment**

To assess dietary intake, food-frequency questionnaires (FFQs) were initially collected in 1986 for 49 935 men (HPFS), in 1980 for 92 468 women (NHS I), and in 1991 for 95 391 women (NHS II), and diet was generally updated every 4 y. For the NHS I, we used a 61-item semiquantitative FFQ at baseline in 1980 (22), which was expanded to ≈130 food items in 1984, 1986, and every 4 y thereafter. For the HPFS and NHS II cohorts, baseline dietary intake was assessed by using a 131-item FFQ (23). For each item, the participants were asked to report their average use over the preceding year. Serving sizes (eg, 1 banana or one-half cup broccoli) were specified for each food in the FFQ. Nine prespecified frequency responses were possible, ranging from never or almost never to 6 times per day. Specific fruit and vegetable items were used to derive total fruit and vegetable intakes as well as intake of composite fruit and vegetable groups, which included cruciferous vegetables, green leafy vegetables, yellow-orange vegetables, citrus fruit, and fruit and vegetables rich in vitamin C. For carotenoid values (α-carotene, β-carotene, β-cryptoxanthin, lutein and zeaxanthin, and lycopene; in µg/d), we used the US Department of Agriculture–National Cancer Institute database that was developed for fruit and vegetables and that includes data on the carotenoid content of tomato-based food products (24–26).

The reproducibility and validity of food intake have been described previously for the HPFS (23, 27) and the NHS I (22, 28, 29). In 127 men from the HPFS cohort, Pearson correlations between the average intake assessed by two 1-wk diet records completed 6 mo apart and the 1986 FFQ completed after the diet records ranged from 0.25 to 0.95 for specific fruit and vegetables, and the median correlations for fruit and vegetables were 0.77 and 0.46, respectively (27). In 173 members of the NHS I cohort, Pearson correlation coefficients, after correction for attenuation due to random error in diet records, between the 1980 FFQ and the means of four 1-wk diet records for fruit and vegetables averaged 0.54 (range: 0.17 for spinach to 0.84 for orange juice) (29). In nonsmoking men and women, intakes of carotenoids correlated reasonably well with specific carotenoid plasma concentrations in a subset of men and women (Pearson correlations ranged between 0.35 and 0.47 for the men and 0.21 and 0.48 for the women) (30).

**Case ascertainment**

On each biennial questionnaire, the participants were asked whether they had been diagnosed with any form of cancer, heart disease, or other medical conditions during the previous 2 y. When permission was received from the case subjects (or next of kin for decedents), medical records and pathology reports were obtained from hospitals and reviewed by study investigators, who were blinded to questionnaire exposure information. Nonrespondents were telephoned in an attempt to confirm the initial cancer report and date of diagnosis. Medical records were requested for reported and deceased glioma cases; ≈88% of glioma diagnoses were confirmed by medical records. When we were unable to obtain medical records, we attempted to corroborate diagnoses of glioma with additional information from the participant, next of kin, by death certificate, or by cross-linking with cancer registries. We only included case subjects for whom a medical record or other confirmation of the cancer was obtained. We included all glioma brain tumors; these included astrocytoma, glioblastoma, oligodendroglioma, ependymoma, and mixed glioma subtypes. Vital status was ascertained through next of kin and the National Death Index (NDI); both methods identify ≥98% of deaths in the cohorts (31). We identified 115 newly diagnosed gliomas between 1986 and 2002 among the men, 165 gliomas among the women in the NHS I between 1980 and 2002, and 16 gliomas among the women in the NHS II between 1991 and 2003.

**Statistical analysis**

Person-time of follow-up was calculated from the date for return of the baseline FFQ (1980 for NHS I, 1986 for HPFS, and 1991 for NHS II) until the date of glioma diagnosis, date of death from any cause, or the end of follow-up (31 December 2002 for HPFS, 31 May 2002 for NHS I, and 31 May 2003 for NHS II), whichever came first. After excluding the participants who reported a history of cancer other than nonmelanoma skin cancer and those with missing information on diet at baseline, the cohorts for analyses included 47 686 (93%) men in the HPFS who were followed for up to 16 y (709 701 person-years of follow-up), 87 662 (95%) women in the NHS I who were followed for up to 22 y, and 94 017 (99%) women in the NHS II who were followed for up to 12 y (2 959 888 total person-years of follow-up among the women). Over the period of follow-up, missing dietary data were carried forward from the previous follow-up cycle from which a participant had an available FFQ.

We estimated the power to detect trends across quartiles for specified incidence rate ratios (RR) in a comparison of the highest with the lowest quartile, assuming a linear relation and fixing the two-tailed α = 0.05 (32). We found a 72% power to detect a RR of 1.5 between the highest and lowest quartiles, a 91% power to detect a RR of 1.7 between the highest and lowest quartiles, and a >99% power to detect a RR of 2.0 between the highest and lowest quartiles.

Baseline dietary intakes were determined by the 1986 FFQ for men in the HPFS, the 1980 FFQ for women in NHS I, and the 1991 FFQ for women in NHS II. For women in the NHS I, we also considered determining baseline dietary intakes with the expanded 1984 questionnaire; however, starting follow-up in 1984 resulted in a reduction in the total number of cases (n = 124 compared with 165), because only 88% of the cohort participants who responded to the 1980 FFQ responded to the 1984 FFQ and because we have 4 fewer years of follow-up. We divided the cohorts by quintiles of fruit, vegetable, and carotenoid intakes. Furthermore, we examined different fruit and vegetable subgroups to represent foods rich in certain nutrients and phytochemicals. Cox proportional hazards models for failure-time data were used to estimate the incidence RRs and 95% CIs for glioma risk and to simultaneously adjust for age (1 y) and total caloric intake, which minimizes extraneous variation introduced by underreporting or overreporting in the FFQ (33). Additional adjustment for potential risk factors, including total meat intake (which consisted of intakes of processed meats; bacon; hot dogs; hamburger; beef, pork, or lamb as a sandwich or mixed dish;
beef, pork, or lamb as a main dish; chicken with skin; and chicken without skin; in quintiles), alcohol consumption (0, 0.1–1.4, 1.5–4.9, 5.0–29.9, or ≥30.0 g/d), coffee consumption (0, ≤1, 2–3, or ≥4 cups/d), pack-years of cigarette smoking history (<10, 10–24, 25–44, or ≥45 pack-years), current smoking; processed meat intake (consisted of processed meats, bacon, and hot dogs; in quintiles), total intake of vitamins C or E (mg/d; energy-adjusted vitamin intake from diet and vitamin supplement; continuous), multivitamin supplement use (yes or no), state of residence in the United States (west, midwest, south, and northeast), body mass index (in kg/m²): 18.0–22.9, 23.0–24.9, 25.0–26.9, 27.0–29.9, or ≥30.0), height (in inches and quintiles), type of profession [among men only; pharmacist, specialist (optometrist or podiatrist), physician, veterinarian, or dentist], and reproductive factors (status and age at menopause: premenopausal; postmenopausal, aged <45 y; postmenopausal, aged 45–49 y; postmenopausal, aged 50–55 y; or postmenopausal, aged ≥55 y), did not significantly change the associations of fruit and vegetable intake with glioma risk. Because of the relative homogeneity of the population of the male health professionals and female nurses, it was unnecessary to control for education or socioeconomic status.

In addition to evaluating the diet at baseline (1980 in NHS I, 1986 in HPFS, and 1991 in NHS II), we examined the relation between the intake of fruit, vegetables, and carotenoids and the risk of glioma by updating baseline dietary intakes with dietary intakes from subsequent questionnaires (in 1984, 1986, 1990, 1994, and 1998 in NHS I; 1990, 1994, and 1998 in HPFS; and 1995 and 1999 in NHS II). In these analyses, we assessed glioma risk in relation to the cumulative average of dietary intakes calculated from all of the preceding dietary questionnaires. For example, in the HPFS, dietary data from the 1986 FFQ was used for follow-up from 1986 to 1990; dietary data from the 1990 FFQ was used for follow-up from 1990 to 1994; data from the 1994 FFQ was used for follow-up from 1994 to 1998; and data from the 1998 FFQ was used for follow-up from 1998 to 2002. The use of cumulative averages may reduce within-person subject variation and better represent long-term average intake. We also examined the relation between recent intake of fruit and vegetables and the risk of glioma by updating diet with the most recent dietary questionnaire. In addition to dietary factors, all covariates were assessed at baseline and were repeatedly assessed from subsequent questionnaires and updated (similar to dietary data) in cumulative average, simple update, and lag analyses. Details of both of these methods are described elsewhere (33, 34). To minimize the possibility that baseline total fruit and vegetable intake may have been altered because of preclinical disease or for other reasons, an analysis excluding the first 2 y of follow-up was performed by using baseline total fruit and vegetable intake. To evaluate time from dietary exposure to glioma diagnosis, we also conducted a 2–6–y lag analysis (because diet was updated every 4 y) using fruit and vegetable intake over each 2–y follow-up cycle. For example, in the HPFS, dietary data from the 1986 FFQ was used for follow-up from 1988 to 1990; dietary data from the 1986 FFQ was used for follow-up from 1990 to 1992; data from the 1990 FFQ was used for follow-up from 1992 to 1994; data from the 1990 FFQ was used for follow-up from 1994 to 1996; data from the 1994 FFQ was used for follow-up from 1996 to 1998; data from the 1994 FFQ was used for follow-up from 1998 to 2000; and data from the 1998 FFQ was used for follow-up from 2000 to 2002.

Additional analyses were restricted by tumor histology [astrocytoma (ICD-O: 94003, 94013, 94113, 94103, 94203, 94213) or glioblastoma (ICD-O: 94403, 94413, 94423)] or tumor site [frontal (ICD-9: 191.1) or temporal (ICD-9: 191.2)]. Tests of linear trend for increasing categories of fruit, vegetable, and carotenoid intakes were conducted with the use of Cox proportional hazards regression by assigning the median values for each and treating those as a single continuous variable. Tests for (multiplicative) interaction were performed by examining stratum-specific estimates and formally with the use of likelihood ratio tests. The age-standardized expected number of cases was calculated by using the 5-y age-specific incidence rate of brain cancer obtained from the Surveillance, Epidemiology, and End Results registry, 1990–2003, and multiplying by the number of person-years in each 5-y age group in the cohort.

Because of the small number of glioma cases observed in the NHS II, the NHS I and NHS II cohorts were combined; the results in the women reflect the pooled estimates of the 2 cohorts. Before pooling with the use of a meta-analysis, tests of heterogeneity of the main exposures by cohort were performed by using the Q statistic, and data were pooled by using a random-effects model for the log of the RR (35); no statistically significant heterogeneity was observed. All reported P values are two-tailed. Statistical analyses were performed by using SAS software version 8.2 (SAS Institute Inc, Cary, NC).

RESULTS

At baseline, men and older women (from NHS I) with a high fruit and vegetable intake were less likely to smoke or drink alcohol or coffee than were individuals who consumed few fruit and vegetables (Table 1). In contrast, younger women (from NHS II) with a high fruit and vegetable intake were more likely to drink alcohol than were those with a low fruit and vegetable intake. Women with a high vegetable intake were more likely to eat meat than those with a low vegetable intake, but intake of meat did not vary across categories of fruit and vegetable intake for men. Intakes of vitamins C and E and multivitamin supplement use were higher among frequent consumers of fruit and vegetables.

The mean age of glioma cases was 65.5 y for men and 60.8 y and 40.6 y for women in the NHS I and NHS II, respectively (Table 2). We collected information on glioma histology or location for 76% (87 cases) of men and 82% (149 cases) of women. For both men (80%) and women (65%), glioblastoma was the most common histologic type [similar to CBTRUS data (1)]; anaplastic astrocytomas accounted for ≈8% and 23% of all cases in men and women, respectively. Glioma was most commonly found in the frontal (28%) and temporal lobe (22%).

After adjustment for age and total caloric intake, we observed no significant overall association between cumulative updated total fruit and vegetable intake and the risk of glioma (Table 3). Risk estimates did not change appreciably after additional inclusion of other covariates, including, but not limited to, total meat intake, alcohol consumption, coffee consumption, and smoking, and increasing age was the strongest risk factor for glioma risk among the men and women (RR for men: 4.7; RR in a comparison of 60–64-y-old women with those <40-y-old: 9.9). No significant correlation was observed between updated intake of fruit, vegetables, cruciferous vegetables, yellow-orange vegetables,
TABLE 1
Age-standardized baseline characteristics by fruit and vegetable intake among men in the Health Professionals Follow-Up Study (HPFS, 1986) and women in the Nurses’ Health Study I (NHS I, 1980) and NHS II (NHS II, 1991) \(^1\)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruit and vegetable intake (quintiles)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of participants</td>
<td>9206</td>
<td>9732</td>
<td>9421</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>52.5 ± 9.4 (^2)</td>
<td>54.7 ± 9.7</td>
<td>55.8 ± 9.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height (in)</td>
<td>69.9 ± 3.4</td>
<td>70.1 ± 3.2</td>
<td>70.1 ± 3.7</td>
<td>0.003</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>25.7 ± 3.2</td>
<td>26.0 ± 3.1</td>
<td>25.5 ± 3.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Past smoker (%)</td>
<td>42.7</td>
<td>43.8</td>
<td>44.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>16.2</td>
<td>8.9</td>
<td>6.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cigarette smoking (pack-years) (^3)</td>
<td>29.1 ± 20.3</td>
<td>24.6 ± 19.4</td>
<td>22.8 ± 18.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Daily dietary intakes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit and vegetables (servings)</td>
<td>2.4 ± 0.7</td>
<td>5.2 ± 0.4</td>
<td>10.6 ± 3.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total meat (servings) (^4)</td>
<td>1.3 ± 0.7</td>
<td>1.3 ± 0.7</td>
<td>1.3 ± 0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alcohol (g)</td>
<td>12.0 ± 16.5</td>
<td>11.5 ± 15.5</td>
<td>10.4 ± 14.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Coffee (cups)</td>
<td>1.5 ± 1.7</td>
<td>1.3 ± 1.5</td>
<td>1.1 ± 1.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin C (mg) (^5)</td>
<td>309 ± 434</td>
<td>414 ± 457</td>
<td>576 ± 500</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin E (mg) (^5)</td>
<td>43.4 ± 90.4</td>
<td>48.0 ± 89.4</td>
<td>61.4 ± 98.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Multivitamin use (%)</td>
<td>57.9</td>
<td>62.3</td>
<td>65.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Women, NHS I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of participants</td>
<td>17 696</td>
<td>17 569</td>
<td>17 515</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>45.2 ± 7.0</td>
<td>46.6 ± 7.1</td>
<td>48.0 ± 7.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height (in)</td>
<td>64.3 ± 3.3</td>
<td>64.5 ± 3.1</td>
<td>64.5 ± 3.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>24.4 ± 4.4</td>
<td>24.5 ± 4.4</td>
<td>24.6 ± 4.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Past smoker (%)</td>
<td>23.5</td>
<td>28.4</td>
<td>31.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>40.0</td>
<td>26.4</td>
<td>21.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cigarette smoking (pack-years) (^3)</td>
<td>24.2 ± 17.0</td>
<td>19.7 ± 16.0</td>
<td>17.9 ± 16.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Daily dietary intakes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit and vegetables (servings)</td>
<td>1.7 ± 0.5</td>
<td>3.7 ± 0.3</td>
<td>7.3 ± 2.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total meat (servings) (^4)</td>
<td>1.3 ± 0.7</td>
<td>1.4 ± 0.7</td>
<td>1.5 ± 0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alcohol (g)</td>
<td>7.3 ± 12.0</td>
<td>6.1 ± 10.0</td>
<td>5.7 ± 9.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Coffee (cups)</td>
<td>2.5 ± 2.1</td>
<td>2.3 ± 2.0</td>
<td>2.1 ± 2.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin C (mg) (^5)</td>
<td>221 ± 456</td>
<td>294 ± 482</td>
<td>416 ± 588</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin E (mg) (^5)</td>
<td>30.7 ± 96.8</td>
<td>33.2 ± 91.6</td>
<td>43.0 ± 99.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Multivitamin use (%)</td>
<td>28.6</td>
<td>34.4</td>
<td>39.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Women, NHS II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of participants</td>
<td>18 712</td>
<td>19 121</td>
<td>18 068</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age (y)</td>
<td>36.2 ± 4.8</td>
<td>36.6 ± 4.7</td>
<td>36.9 ± 4.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height (in)</td>
<td>64.8 ± 2.6</td>
<td>64.9 ± 2.6</td>
<td>65.0 ± 2.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>24.8 ± 5.7</td>
<td>24.5 ± 5.2</td>
<td>24.6 ± 5.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Past smoker (%)</td>
<td>19.3</td>
<td>22.5</td>
<td>24.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>16.9</td>
<td>11.1</td>
<td>10.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cigarette smoking (pack-years) (^3)</td>
<td>13.5 ± 9.4</td>
<td>11.7 ± 8.5</td>
<td>11.0 ± 8.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Daily dietary intakes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit and vegetables (servings)</td>
<td>2.0 ± 0.6</td>
<td>4.6 ± 0.4</td>
<td>9.6 ± 2.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total meat (servings) (^4)</td>
<td>1.1 ± 0.6</td>
<td>1.3 ± 0.6</td>
<td>1.4 ± 0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alcohol (g)</td>
<td>2.7 ± 6.1</td>
<td>3.2 ± 6.0</td>
<td>3.4 ± 6.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Coffee (cups)</td>
<td>1.2 ± 1.7</td>
<td>1.3 ± 1.6</td>
<td>1.3 ± 1.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin C (mg) (^5)</td>
<td>199 ± 336</td>
<td>256 ± 318</td>
<td>333 ± 314</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin E (mg) (^5)</td>
<td>23.8 ± 65.3</td>
<td>24.9 ± 58.0</td>
<td>29.1 ± 58.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Multivitamin use (%)</td>
<td>36.3</td>
<td>44.5</td>
<td>51.0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\(^1\) Baseline was 1986 for the men in the HPFS, 1980 for the women in the NHS I, and 1991 for the women in the NHS II. Of note, for the women, the 1980 food-frequency questionnaire included fewer fruit and vegetable items than did the 1991 food-frequency questionnaire.

\(^2\) ± SD (all such values).

\(^3\) Pack-years are calculated for current and past smokers.

\(^4\) Total meat consists of processed meats, bacon, hot dogs, hamburger, beef, pork, or lamb as a sandwich or mixed dish; beef, pork, or lamb as a main dish; chicken with skin; and chicken without skin.

\(^5\) Energy-adjusted vitamin intake from the diet and vitamin supplements.

Green-leafy vegetables, citrus fruit, or fruit and vegetables rich in vitamin C and glioma risk.

Results with the use of baseline or most recent total, fruit, vegetables, or composite fruit and vegetable groups were similar to those that used cumulative updated diet, with one exception: cruciferous vegetable intake (data not shown). At baseline, we observed a marginally statistically significant decrease in glioma risk in the women with the highest cruciferous vegetable intake compared with those with the lowest intake (RR in a comparison of the highest with the lowest intake quintile: 0.53; 95% CI: 0.33,
For trend

However, no significant association was observed in the men (RR in a comparison of the highest and lowest quintiles: 1.29; 95% CI: 0.73, 2.26; P for trend = 0.60).

To account for changes in diet because of preclinical manifestations of disease or for other reasons, we conducted additional analyses that excluded all cases of glioma diagnosed within the first 2 y of follow-up. On the basis of 268 glioma cases (2,190,548 person-years), no significant associations were observed for fruit, vegetables, or other fruit and vegetable subgroups (data not shown). Similarly, removing men or women who reported having changed their fruit or vegetable intake in the previous decade (on the baseline questionnaire) resulted in associations similar to those observed with the updated dietary intake (data not shown).

To evaluate time from exposure to glioma diagnosis, we conducted a 2–6-y lag analysis (see Subjects and Methods); no significant association with glioma risk for fruit, vegetables, or other fruit and vegetable subgroups was observed (data not shown).

To further explore specific nutrients in fruit and vegetables, we examined the association between glioma risk and updated dietary intake of the major carotenoids for the men and women. No material relation was observed between the updated intake of α-carotene, β-carotene, β-cryptoxanthin, lutein and zeaxanthin, and lycopene and the risk of glioma (Table 4). For lycopene, baseline intake was associated with a suggestive increased glioma risk in the women; the RRs for glioma in the multivariate model were 1.23 for the 2nd quintile, 1.63 for the 3rd quintile, 1.11 for the 4th quintile, and 1.66 (95% CI: 1.04, 2.67) for the 5th quintile (P for trend = 0.04).

Intake of individual fruit and vegetables that constitute the composite fruit and vegetable food groups was not appreciably associated with glioma risk (data not shown). The only exception was intake of cabbage, cauliflower, or Brussels sprouts in the women: the RR of glioma in a comparison of the highest with the lowest tertile of intake was 0.60 (95% CI: 0.40, 0.91).

Additional analyses conducted among more homogeneous cancer subgroups showed similar null results between updated total fruit and vegetable intake for the other fruit and vegetable subgroups and the risk of astrocytoma or glioblastoma (data not shown). Furthermore, no significant relation between dietary intake and glioma risk was observed by anatomic site (frontal or temporal lobe, separately) or after excluding glioma cases that were not confirmed by pathology records (data not shown).

The association between updated total fruit and vegetable intake and glioma risk was examined across strata of cigarette smoking status (never or ever) and age (median) in the men and women (data not shown); there was no evidence that the association was significantly modified by either P for interaction for smoking status: 0.67 and 0.87; P for interaction of age: 0.64 and 0.62, for men and women, respectively). Furthermore, there was no evidence that the association was significantly modified by meat intake (total or processed; data not shown).

### TABLE 2
Characteristics of glioma cases among men in the Health Professionals Follow-Up Study (HPFS: 1986 to 2002) and women in the Nurses’ Health Study I (NHS I: 1980 to 2002) and NHS II (1991 to 2003)

<table>
<thead>
<tr>
<th>Glioma characteristic</th>
<th>Men, HPFS</th>
<th>Women, NHS I</th>
<th>Women, NHS II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude incidence rate (no. per 100,000 person-years)</td>
<td>16</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Observed number of cases</td>
<td>115</td>
<td>165</td>
<td>16</td>
</tr>
<tr>
<td>Expected number of cases</td>
<td>111</td>
<td>170</td>
<td>45</td>
</tr>
<tr>
<td>Observed:expected</td>
<td>1.04 (0.86, 1.23)</td>
<td>0.97 (0.83, 1.12)</td>
<td>0.36 (0.20, 0.55)</td>
</tr>
<tr>
<td>Age at diagnosis (y)</td>
<td>65.5 ± 9.9</td>
<td>60.8 ± 8.5</td>
<td>40.6 ± 6.5</td>
</tr>
<tr>
<td>Median</td>
<td>65.6</td>
<td>61.4</td>
<td>38.8</td>
</tr>
<tr>
<td>Range</td>
<td>43.5–85.1</td>
<td>38.8–78.5</td>
<td>28.1–49.1</td>
</tr>
<tr>
<td>Histology (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astrocytoma</td>
<td>94.3</td>
<td>89.1</td>
<td>75.0</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>80.5</td>
<td>66.4</td>
<td>50.0</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>0</td>
<td>3.6</td>
<td>16.7</td>
</tr>
<tr>
<td>Ependymoma</td>
<td>1.1</td>
<td>0.7</td>
<td>0</td>
</tr>
<tr>
<td>Mixed glioma</td>
<td>1.1</td>
<td>2.2</td>
<td>8.3</td>
</tr>
<tr>
<td>Unknown or missing</td>
<td>3.5</td>
<td>4.4</td>
<td>0</td>
</tr>
<tr>
<td>Anatomical site (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal</td>
<td>27.6</td>
<td>26.3</td>
<td>41.7</td>
</tr>
<tr>
<td>Temporal</td>
<td>20.7</td>
<td>23.4</td>
<td>0</td>
</tr>
<tr>
<td>Parietal</td>
<td>12.6</td>
<td>16.1</td>
<td>16.7</td>
</tr>
<tr>
<td>Occipital</td>
<td>5.7</td>
<td>0.7</td>
<td>0</td>
</tr>
<tr>
<td>Multilobar</td>
<td>6.9</td>
<td>11.7</td>
<td>25.0</td>
</tr>
<tr>
<td>Other</td>
<td>10.3</td>
<td>3.7</td>
<td>0</td>
</tr>
<tr>
<td>Unknown or missing</td>
<td>16.2</td>
<td>18.1</td>
<td>16.6</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White (%)</td>
<td>94.7</td>
<td>94.2</td>
<td>92.6</td>
</tr>
</tbody>
</table>

1 Number of observed cases confirmed by pathology reports: HPFS = 87, NHS I = 137, NHS II = 12.
2 Age-specific incidence rate of brain cancer obtained from Surveillance, Epidemiology, and End Results (SEER) registry, 1990 to 2003.
3 95% CI in parentheses (all such values).
4 Cerebellum, brain stem, corpus callosum, tectum, or ventricle.
DISCUSSION

In our study of fruit and vegetable consumption in well-defined, large prospective cohorts of men and women with a large number of glioma cases and validated, updated dietary information, we found no significant overall associations between consumption of total fruit or vegetables and glioma risk in men and women. Similarly, there was no evidence of any appreciable benefit from any of the specific subgroups of fruit and vegetables. Overall, no significant association was observed between dietary intakes of \( \beta \)-carotene, \( \beta \)-cryptoxanthin, lutein and zeaxanthin, and lycopene and the risk of glioma.

The biological rationale for the intake of fruit and vegetables in glioma prevention relates largely to the potential ability of several dietary constituents of fruit and vegetables to inhibit nitrosation. Nitrosation, the endogenous formation of NOC from dietary precursors, is a complex process that is dependent on the presence of dietary NOC precursors including nitrate and nitrite, the presence of bacteria and nitrosation catalysts or inhibitors, gastric pH, and other physiologic variables (36–39). The endogenous nitrosation reaction may be blocked by the presence of nitrogen scavengers, such as vitamins C and E, phenolics, or antioxidants in the diet (16–18). Both vitamins C and E are...
powerful reducing agents and are oxidized to reduce nitrous acid to nitric oxide (17). The presence of these vitamins and other nutrients, including carotenoids, from intake of fruit and vegetables in the diet may decrease or inhibit the endogenous formation of NOC or may act directly as antioxidants protecting against oxidative stress, free-radical reactions, DNA or cellular damage, and lipid peroxidation (19–21) and, consequentially, reduce the risk of glioma.

Observational studies of intakes of fruit and vegetables and their dietary constituents on the risk of glioma have been inconclusive (2–14) (Table 5). Several case-control studies report null results for intakes of fruit (4, 5, 7, 11), vegetables (4, 5, 7, 11), fruit or vegetable subgroups (5, 6, 11–13), and individual fruit and vegetable food items (3, 5, 6, 8). Two case-control studies by Hu et al (9, 10), which used the same study population, reported significant inverse associations between intakes of fruit and fresh vegetables and glioma or meningioma risk. Chen et al (6) reported a marginally statistically significantly reduced risk of glioma among those in the highest quartile of vegetable and dark yellow vegetable consumption compared with those in the lowest quartile and an ≈50% reduction in glioma risk with increased \( \alpha \)- and \( \beta \)-carotene intake. In a recent case-control study, a reduced risk of glioma was observed for higher antioxidant index (\( P \) for trend = 0.002), carotenoids (\( \alpha \)- and \( \beta \)-carotene combined; \( P \) for trend = 0.02), and \( \beta \)-carotene (\( P \) for trend = 0.04) (14). In the single prospective study, during 6 y of follow-up, 21 of 34 000 Seventh-Day Adventist participants developed glioma (2). No association was observed between the derived fruit index [canned or frozen fruit, dried fruit, fresh citrus fruit, and other fresh fruit (apples, bananas, and pears)], fresh citrus fruit, or unsweetened or sweetened real fruit juice and glioma risk.

The inverse association observed for cruciferous vegetables among the women may be due to chance, because it was not observed in the men and was marginally statistically significant only with the baseline analyses but not with other analytic approaches. Alternatively, it is possible that cruciferous vegetables, which include broccoli, cabbage, cauliflower, Brussels sprouts, and kale, play a role in glioma etiology, because experimental studies (40–42) have identified several compounds found in cruciferous vegetables, eg, isothiocyanate sulforaphane, that can induce phase 2 detoxifying enzymes in vitro and stimulate metabolism of drugs and other xenobiotics in humans (43–45).
**TABLE 5**

Summary of selected epidemiologic studies of the relation between intakes of dietary fruit, vegetables, and carotenoids and brain tumors.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study location and time period</th>
<th>Subjects</th>
<th>Cases, proxy %</th>
<th>Risk estimate (95% CI)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mills et al (1989)</td>
<td>California Seventh-Day Adventist cohort (USA), 1976–1982</td>
<td>Glioma: 21 M and F 6 yr of follow-up</td>
<td></td>
<td>RR = 0.85 (0.28, 2.60) RR = 0.92 (0.37, 2.37) RR = 1.65 (0.59, 5.77) RR = 0.29 (0.01, 1.59)</td>
<td>Fruit index, ≥1 time/d Citrus fruit, ≥1 time/wk Unsweetened fruit juice, ≥1 time/wk Sweetened fruit juice, ≥1 time/wk</td>
</tr>
<tr>
<td>Preston-Martin and Mack (1991)</td>
<td>Los Angeles, CA (USA), 1980–1984</td>
<td>Glioma: 202 M 202 neighborhood control subjects</td>
<td>0</td>
<td>0.8 (0.4, 1.6)</td>
<td>Citrus fruit, &gt;5 times/wk</td>
</tr>
<tr>
<td>Boeing et al (1993)</td>
<td>Germany, 1987–1988</td>
<td>Glioma: 115 M and F 418 population control subjects</td>
<td>23</td>
<td>1.1 (0.6, 1.9) 0.9 (0.5, 1.7)</td>
<td>Fruit, highest tertile Vegetables, highest tertile</td>
</tr>
<tr>
<td>Giles et al (1994)</td>
<td>Victoria Province, Australia, 1987–1991</td>
<td>Glioma: 166 M and F 409 population control subjects</td>
<td>≥50</td>
<td>1.51 (0.95, 2.39) 0.69 (0.36, 1.31) 1.01 (0.62, 1.63) 0.53 (0.29, 0.95) 0.97 (0.60, 1.56) 0.70 (0.39, 1.26)</td>
<td>M, fruit, highest tertile F, fruit, highest tertile M, vegetables, highest tertile F, vegetables, highest tertile M, β-carotene, highest tertile F, β-carotene, highest tertile</td>
</tr>
<tr>
<td>Blowers et al (1996)</td>
<td>Los Angeles, CA (USA), 1986–1988</td>
<td>Glioma: 94 F 94 neighborhood control subjects</td>
<td>0</td>
<td>1.3 (0.5, 3.0) 1.3 (0.5, 3.2) 1.7 (0.7, 4.3) 0.2 (0.1, 0.7) 0.5 (0.2, 1.3)</td>
<td>Fruit, highest quartile Vegetables, highest quartile Citrus fruit or juice, highest quartile Bell peppers, highest quartile Carrots, highest quartile</td>
</tr>
<tr>
<td>Kaplan et al (1997)</td>
<td>Tel Hashomer, Israel, 1987–1991</td>
<td>Glioma (59) and meningioma; 139 M and F 278 friend and hospital control subjects</td>
<td>22</td>
<td>1.76 (1.03, 3.01) 2.01 (1.17, 3.46) 0.91 (0.55, 1.52) 0.65 (0.37, 1.12)</td>
<td>Fruit, ≥104.1 g/d Fruit, high vitamin C, ≥195.1 g/d Vegetables, ≥84.1 g/d Carrots, ≥23 g/d</td>
</tr>
<tr>
<td>Lee et al (1997)</td>
<td>San Francisco Bay, CA (USA), 1991–1994</td>
<td>Glioma: 434 M and F 434 population control subjects</td>
<td>46</td>
<td>NA</td>
<td>Fruit or vegetables high in vitamin A or C</td>
</tr>
<tr>
<td>Hu et al (1998)</td>
<td>Northeast China, 1989–1995</td>
<td>Glioma: 218 M and F 436 hospital control subjects</td>
<td>0</td>
<td>0.28 (0.16, 0.51) 0.51 (0.29, 0.89)</td>
<td>Fruit, ≥46 kg/y Vegetables, ≥125 kg/y</td>
</tr>
<tr>
<td>Hu et al (1999)</td>
<td>Northeast China, 1993–1995</td>
<td>Glioma (73) and meningioma; 129 M and F 258 hospital control subjects</td>
<td>0</td>
<td>0.15 (0.1, 0.4) 0.18 0.29 (0.1, 0.7) 0.34 2.54 (1.2, 5.6)</td>
<td>Fruit, highest quartile Fruit, highest quartile Fresh vegetables, highest quartile Fresh vegetables, highest quartile Salted vegetables, highest quartile</td>
</tr>
<tr>
<td>Chen et al (2002)</td>
<td>Nebraska (USA), 1988–1993</td>
<td>Glioma: 236 M and F 449 population control subjects</td>
<td>76</td>
<td>1.0 (0.6, 1.7) 0.5 (0.3, 1.0) 0.7 (0.4, 1.2) 0.6 (0.3, 1.0) 1.2 (0.7, 2.0) 0.7 (0.4, 1.3) 0.5 (0.3, 0.8) 0.5 (0.3, 0.9) 0.7 (0.4, 1.3) 1.2 (0.7, 2.1) 0.9 (0.5, 1.6)</td>
<td>Citrus fruit, highest quartile Vegetables, highest quartile Dark green vegetables, highest quartile Dark yellow vegetables, highest quartile Tomatoes, highest quartile High-nitrate vegetables, highest quartile α-Carotene, highest quartile γ-Carotene, highest quartile β-Cryptoxanthin, highest quartile Lutein, highest quartile Lycopene, highest quartile</td>
</tr>
</tbody>
</table>

(Continued)
The suggestive elevation in risk of glioma for lycopene at baseline did not persist in the women when we examined cumulative, updated average intake, which took into account cooked and raw tomato-based sources of lycopene (the baseline NHS I questionnaire included intake of only fresh tomatoes). With the cumulatively updated data, we were able to enhance the precision of dietary assessments, account for changes in consumption over time, and reduce the potential for within-person misclassification of intake. Overall, we observed no significant relation between 5 major carotenoids and glioma risk.

The strengths of our study included its large sample size, the prospective design, long follow-up, and detailed and updated information on fruit and vegetable consumption, with up to 22 fruit items and 38 vegetable items. The prospective design precludes recall bias, and selection bias is minimized by the high rate of follow-up over a long period of time. No proxies were needed, because information on diet was obtained before the occurrence of disease. The availability of repeated dietary measures in the cohorts permitted a consideration of early (baseline), most recent (simple update), and long-term (cumulative updated and restricted analyses) dietary intake. We cannot exclude measurement error due to self-reported diet as a contributor to the lack of associations in the current study; however, we previously showed the accuracy of self-reported dietary intake in these cohorts, and the repeated assessment of intake may reduce between-person subject variation and better represent long-term average intake. Misclassification of disease status may occur because of undetected or underreported cancer due to misdiagnosis. We cannot exclude the possibility that some cancers may have been missed in the study; however, it is unlikely given the comprehensive method of disease surveillance in the cohorts. Furthermore, the results from the secondary analyses, which excluded glioma cases not confirmed by pathology records, were similar to those when all cases were considered.

In conclusion, our study provides limited support for the hypothesis that dietary intake of fruit, vegetables, and carotenoids with antioxidant properties and the potential ability to inhibit the endogenous formation of NOC may reduce the risk of adult glioma. High consumption of total fruit and vegetables, fruit and vegetable subgroups, and carotenoids were not significantly related to the risk of glioma in our large US cohorts.

We thank Walter Willett for his valuable advice and Barbara Vericker and Barbara Egan for their assistance.

ELG, MJS, and DSM contributed to the study design. CNH was responsible for writing and revising the manuscript. ELG, BR, MJS, and DSM provided substantive editorial comments on manuscript drafts. None of the authors had any personal or financial conflicts of interest.

REFERENCES

Zinc during and in convalescence from diarrhea has no demonstrable effect on subsequent morbidity and anthropometric status among infants <6 mo of age

Christa L Fischer Walker, Zulfiqar A Bhutta, Nita Bhandari, Telahun Teka, Farhana Shahid, Sunita Taneja, Robert E Black, and the Zinc Study Group

ABSTRACT
Background: Preventing illness and improving growth in the first 6 mo of life is critical to reducing infant mortality. Zinc given for 14 d at the start of diarrhea has been shown to decrease the incidence and prevalence of diarrhea and pneumonia and improve growth in the 2–3 mo after, but no trial has been done in infants <6 mo of age.

Objective: This study sought to assess the effect of 14 d of zinc supplementation on subsequent morbidity and growth among infants 1–5 mo of age living in Pakistan, India, and Ethiopia.

Design: Infants with acute diarrhea were randomly assigned to receive zinc (10 mg/d; n = 538) or placebo (n = 536) for 2 wk. Weekly follow-up visits were conducted for 8 wk after the diarrhea episode. Incidence and prevalence of diarrhea and prevalence of respiratory infections including pneumonia were compared between the groups. Changes in weight, length, and corresponding z scores during the 8 wk of follow-up were also compared.

Results: One thousand seventy-four infants were enrolled at the start of follow-up. The groups did not differ significantly in the proportion of infants with at least one episode of diarrhea or respiratory infections. Infants who received zinc had more days of diarrhea (rate ratio = 1.20) than did the infants who received placebo. The groups had similar prevalences of pneumonia and overall respiratory infections. No significant differences in the mean changes in weight-for-age, length-for-age, and weight-for-length z scores were observed between the groups overall or in stratified analyses.

Conclusion: Young infants do not appear to benefit from 2 wk of zinc, unlike what has been observed among older children. Am J Clin Nutr 2007;85:887–94.

KEY WORDS Zinc, infants, growth, diarrhea management, pneumonia, diarrhea

INTRODUCTION
Diarrhea is the second leading cause of death among children <5 y of age, accounting for 18% of all child deaths (1). Although in recent decades the use of Oral Rehydration Solution has led to a decrease in the case fatality rate, diarrhea incidence rates have remained unchanged in the developing world (2). Malnutrition is an underlying risk factor for 61% of all childhood deaths associated with diarrhea (1). In addition to causing mortality, diarrhea is an important determinant of growth faltering in the developing world (3)

Zinc has been shown to be an effective treatment for diarrhea in young children, shortening the duration and severity of the diarrhea episode, and is now recommended by the World Health Organization (WHO) and UNICEF as part of diarrhea therapy for all children aged <5 y (4, 5). When given for 10–14 d during and after a diarrhea episode, zinc has benefits for the subsequent 2–3 mo in decreasing the prevalence of diarrhea [odds ratio (OR): 0.66, 95% CI: 0.52, 0.83] and the incidence of pneumonia (OR: 0.74; 95% CI: 0.40, 1.37) (6–10). Zinc for diarrhea treatment has also been shown to help children maintain their weight during a diarrhea episode and to improve growth in the weeks after an episode (11–13). The prevention of diarrhea and pneumonia is critical, especially in areas where limited access and financial constraints prevent prompt treatment, as is the case in much of the developing world (14, 15).

We conducted a randomized, placebo-controlled trial to assess the effect of zinc on diarrhea among infants 1–5 mo of age in Pakistan, India, and Ethiopia, where there are high rates of infant mortality, diarrhea, and stunting (16–19). We previously reported that there was no effect of zinc on the duration and severity of the treated diarrhea episode among these young infants (20). Herein we report the effect of zinc on morbidity and growth during 8 wk of follow-up after the index diarrhea episode. The current study is the first to assess the effects of zinc on subsequent morbidity and growth exclusively in infants <6 mo of age.

1 From the Johns Hopkins Bloomberg School of Public Health, Department of International Health, Baltimore, MD (CLFW and REB); The Aga Khan University, Department of Pediatrics and Child Health, Karachi, Pakistan (ZAB and FS); Society for Applied Studies, New Delhi, India (NB and ST); and Addis Ababa University Faculty of Medicine, Department of Pediatrics, Addis Ababa, Ethiopia (TT).
2 This study was made possible by the generous support of the American people through the United States Agency for International Development (USAID). The contents do not necessarily reflect the views of USAID or the United States Government.
3 Supported by the Johns Hopkins Family Health and Child Survival and Global Research Activity Cooperative Agreement with the US Agency for International Development. The zinc and placebo supplements were provided by the World Health Organization.
4 Reprints not available. Address correspondence to CL Fischer Walker, Johns Hopkins Bloomberg School of Public Health, 615 North Wolfe Street, Room E8031, Baltimore, MD 21218. E-mail: cfischer@jhsph.edu.
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Accepted for publication October 20, 2006.
SUBJECTS AND METHODS

We enrolled infants for this randomized, placebo-controlled diarrhea treatment trial and continued follow-up for 8 wk after the initial diarrhea episode to monitor subsequent morbidity and growth. Infants were enrolled from October 2003 through February 2005 from low income districts of Addis Ababa, Ethiopia, Karachi, Pakistan, and New Delhi, India. Complete screening and enrollment details have been described (20). Briefly, we enrolled 1110 infants 1–5 mo of age with <72 h of diarrhea who had no signs of other serious illnesses. Infants were excluded if they presented with a serious underlying illness, such as severe pneumonia or severe malnutrition, or if the infant required an overnight hospital stay. Sample size calculations for all outcome measures including morbidity and anthropometric follow-up were calculated with 80% power assuming a 2-sided $\alpha$ of 0.05.

We enrolled 1110 infants to detect a difference of 0.16 in the length-for-age $z$ (LAZ) score and a 25% decline in diarrhea incidence rate between zinc and placebo groups, assuming a 10% loss to follow-up.

The study was thoroughly explained to the parents or guardians of the eligible infants. The parents were told the purpose, potential benefits, potential risks, and time commitment for participation in the study. If the parent agreed, he or she was asked to sign a written parental permission document outlining the study details. All study methods, including consent procedures and documentation, were approved by the Johns Hopkins Bloomberg School of Public Health Institutional Review Board, the Addis Ababa Faculty of Medicine Ethics Board, the Aga Khan University Faculty of Science Ethical Review Committee, and the Society for Applied Studies Ethical Review Committee.

Infants who met all inclusion and no exclusion criteria and whose parent signed the permission document were enrolled in the study. Enrolled infants were randomly assigned to receive 10 mg zinc sulfate or placebo in the form of a dispersible tablet once per day for 14 d. Each blister pack was prenumbered according to a block randomization scheme conducted at the WHO in Geneva. Numbered packs were then sent to each study site. The randomization code was kept in Geneva until all analyses were complete. Zinc and placebo tablets looked and tasted the same to ensure blinding. All investigators, study personnel, and parents of enrolled infants were blinded from the randomization scheme until data analyses were complete.

A trained doctor or nurse conducted the baseline interview and clinic visit to assess the history of the diarrhea, breastfeeding status before and since the start of the diarrhea episode, and sociodemographic characteristics. Weight was measured by placing the lightly clothed infant on an infant scale and recording to the nearest 100 g. The measurement was recorded after the needle was steady (in India and Ethiopia) or when the digital read display remained unchanged (in Pakistan) for several seconds. The scales were calibrated daily to ensure accuracy. Recumbent length was measured by using an infant length board and recording to the nearest 0.1 cm. All doctors, nurses, and data collectors were trained to measure both length and weight using standard methodology.

The infant was followed up at home or in the clinic every 3 d by a trained data collector until the infant passed <3 loose or watery stools/24 h and had maintained this non-diarrhea state for $\geq48$ h. On the last diarrhea episode follow-up visit the infant’s length and weight were recorded as described above.

Weekly follow-up began 1 wk after the first diarrhea free day and continued for 8 wk. Daily information on morbidity was recorded. In the case where the parent or infant was unavailable or the visit fell on a holiday, the follow-up visit was conducted the following 1–2 d. Every effort was made to retain the infants in the study and find missing enrollees. Only if the infant was not found after daily visits for 7 d was the child dropped from the study. Until the end of the 14-d supplementation period, the data collector asked the mother about compliance with the tablets and verified each verbal report by counting the missing tablets from the blister pack. The data collector asked the mother about any diarrhea or respiratory symptoms during the previous 7 d and assessed the infant for current cough or difficulty breathing. If the infant had cough or difficulty breathing, the data collector counted the infant’s respiratory rate, looked for chest indrawing, and took his or her axillary temperature. The data collector also asked the parent or guardian if the infant had had any other illnesses in the past week or had been to any health care facility for any reason. On the 4th and the 8th weeks of follow-up, the infant’s length and weight were measured as described above. Infants were visited by the same data collector to ensure there was precision in the length and weight measurements. Infants with observed or reported illnesses at the time of follow-up were referred to the study clinic or hospital for appropriate treatment.

Statistical methods

The sex of the infant, age, mean number of children in the household, mother’s education, exclusive breastfeeding status, and presentation with cough or difficulty breathing in addition to diarrhea on enrollment were assessed by group for all infants. Differences in means were assessed with a Student’s $t$ test analysis and differences in proportion by Pearson’s chi-square analysis.

An episode of diarrhea was defined as $\geq1$ d of diarrhea as reported by the caregiver. The episode was not considered over until there were >48 h with no reported diarrhea (21). The proportion of infants with $\geq1$ episode of diarrhea, the proportion of infants with $\geq2$ episodes of diarrhea, and the proportion of infants with $\geq1$ episode of dysentery during the 8 wk of follow-up were calculated.

The diarrhea incidence rate was calculated by dividing the total number of episodes of diarrhea per child by the total number of days of follow-up and multiplying by 30.42 for a monthly rate. Rate ratios (RRs) were then calculated.

Any infant was reported as having a respiratory infection during the past week if the mother reported any one or more of the following signs or symptoms: cough, fast breathing, difficulty breathing, chest indrawing, wheezing, nasal flaring, grunting, congestion, runny nose, or stuffed nose. Pneumonia was defined per the WHO definition (22), ie, as cough and difficult or fast breathing on the day of follow-up visit with a respiratory rate of $\geq60$ breaths/min for infants $<2$ mo of age or $\geq50$ breaths/min for infants aged $\geq2$ mo. Reported cases of pneumonia were considered respiratory infections and only classified as pneumonia if confirmed by an elevated respiratory rate on the day of follow-up. The proportion of infants with $\geq1$ reported respiratory infection and pneumonia episode were compared by group. The presence of any respiratory infection or pneumonia episode was recorded weekly. Prevalence of respiratory infection was calculated by finding the total number of weeks with respiratory symptoms, dividing by the number of weeks of follow-up, and
multiplying by 4.3. Prevalence of pneumonia infection was calculated by finding the total follow-up days where the child met previously described pneumonia criteria, dividing by the number of days of follow-up, and multiplying by 30.42. RRs were calculated.

Mothers were asked to report care-seeking at any health care facility, including a hospital, for each day during the 8 wk of follow-up. Hospitalization was defined as any overnight stay at a health care facility. The proportion of infants taken to a health facility and the proportion hospitalized during the 8 wk of follow-up were calculated for each group.

The proportions of infants with diarrhea, respiratory infection, or pneumonia were assessed by using a multivariate logistic regression, and a difference in rates was assessed with a robust Poisson regression. Before each full regression analyses for all outcomes, interaction between supplementation group and nutritional status at the start of follow-up [weight-for-length (WLZ) score] was assessed. Differences between the zinc and placebo groups for all proportions were determined by a multiple logistic regression analysis after adjustment for diarrhea episodes lasting >7 d, exclusive breastfeeding on enrollment, and the WLZ score at the start of follow-up.

The mean weight (g) and length (cm) at start of follow-up, week 4 of follow-up, and week 8 of follow-up were calculated and plotted by group. t Tests were used to calculate any differences between the zinc and placebo groups for both weight and length at all time points. z Scores were calculated by using the WHO reference population of breastfed infants (23). Whereas we previously used the National Center for Health Statistics/WHO reference population for the z score calculations, for this analysis, z scores were calculated by using the latest WHO recommendation (WHO Child Growth Standards/ANTHRO 2005), which calculates z scores on the basis of exact age and weight or length and compared to the WHO breastfed reference population (24). The mean weight-for-age (WAZ), LAZ, and WLZ scores were calculated at the start of follow-up and after 8 wk of follow-up and compared by group and site.

Baseline z scores were compared by the Student’s t test analysis. The mean change in z scores (WAZ, LAZ, and WLZ) from the start of follow-up until the end of follow-up (week 8) was compared by group by using analysis of variance (ANOVA), with control for the baseline z score. The data were then stratified by baseline WAZ (<–2 WAZ compared with ≥–2 WAZ), LAZ (<–2 LAZ compared with ≥–2 LAZ), breastfeeding status on enrollment (exclusive or not exclusive), and by sex. ANOVA regressions were used to assess differences between zinc and placebo groups for the overall mean change and for each stratified analysis while controlling for sex, WAZ, LAZ, exclusive breastfeeding, and the total days of diarrhea during the follow-up period. Possible interactions between site and treatment group were assessed for each outcome measure. Interaction terms were incorporated into the previously described, and no statistically significant differences were observed (P > 0.20) (results not presented). All analyses were performed by using STATA version 9.0 (Stata Corp, College Station, TX).

RESULTS

A total of 1074 infants began the weekly enrollment. Infants in the zinc group were more likely to be girls and to have been exclusively breastfed before the diarrhea episode than were infants who received placebo; all other characteristics were not statistically different between the 2 groups (Table 1). Of those enrolled, 998 infants completed the 8 wk of follow-up; 32 infants were not fully followed-up because they withdrew from the study. For 46 infants, the initial diarrhea episode lasted longer than 9 d; therefore, the follow-up weeks during that time were excluded. Infants in the zinc group contributed 4200 infant-weeks of observation and infants who received placebo contributed 4169 infant-weeks of observation for the morbidity analyses (Figure 1). One infant in the zinc group and 6 infants in the placebo group were missing final growth data and were therefore excluded from the growth analysis. Thus, 522 infants in the zinc group and 513 infants in the placebo group contributed complete data for the final growth analysis.

Both the proportion of infants with ≥one episode of diarrhea and the proportion of infants with ≥2 episodes of diarrhea were not statistically different between the groups (Table 2). No significant difference between the diarrhea incidence rates of infants in the zinc and placebo groups after control for covariates (RR = 1.02; 95% CI: –0.18, 0.15). Infants in the zinc group had more days of diarrhea during the follow-up period (RR = 1.22; 95% CI: –0.01, 0.36) after control for covariates.

The proportion of infants with ≥1 respiratory infections or pneumonia episodes was not significantly different between the groups (Table 3). The prevalence of respiratory infections (RR = 1.00; 95% CI: –0.09, 0.27) and the prevalence of pneumonia (RR = 1.04; 95% CI: –0.33, 0.49) were also not statistically different. No significant difference in the proportions of infants who were brought to a health care facility or hospitalized overnight at any point during follow-up was observed between the zinc- and placebo-supplemented infants (Table 3).

Weight and length were not significantly different between the zinc and placebo groups at the start of follow-up, week 4, and week 8 (Table 4). Mean weight and length increased linearly from the start of follow-up to week 4 and week 8. WAZ, LAZ, and WLZ scores at baseline were not significantly different between the zinc and placebo groups (Table 5). The average change in z scores per month were calculated for WAZ, LAZ, and WLZ scores. No significant differences in the monthly z score changes were observed between the zinc and placebo groups for WAZ, LAZ, or WLZ as assessed by ANOVA after control for covariates (Table 5). Also, no significant differences in the average change in WAZ, LAZ, or WLZ scores were observed when stratified by WAZ at the start of follow-up, LAZ at the start of follow-up, exclusive breastfeeding at enrollment, or sex (data not shown).

DISCUSSION

Zinc has been proven to be an effective treatment for diarrhea in children aged 6–59 mo and has been shown to decrease the incidence and prevalence of infectious diseases for 2–3 mo after 2 wk of daily zinc (4, 10, 25). However, there is uncertainty about the effect of this approach in young infants. We randomly assigned infants 1–5 mo of age with acute diarrhea to receive either zinc or placebo for 14 d and continued observations for 8 wk after the diarrhea episode. To our knowledge, the current study is the first to look at both the immediate and longer term effects of zinc on morbidity and growth among infants enrolled before 6 mo of...
TABLE 1
Baseline characteristics by site and supplementation group

<table>
<thead>
<tr>
<th>Selected baseline characteristics of infants in weekly follow-up</th>
<th>All sites</th>
<th>Pakistan</th>
<th>India</th>
<th>Ethiopia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zinc (n = 538)</td>
<td>Placebo (n = 536)</td>
<td>Zinc (n = 273)</td>
<td>Placebo (n = 270)</td>
</tr>
<tr>
<td>Boys (%)</td>
<td>49.1 (44.8, 53.4)\textsuperscript{2, 3}</td>
<td>56.7 (52.4, 61.0)</td>
<td>30.2 (26.4, 34.4)\textsuperscript{2}</td>
<td>47.6 (41.6, 53.7)\textsuperscript{2}</td>
</tr>
<tr>
<td>Age at end of 3 d follow-up (mo)</td>
<td>3.4 ± 1.4\textsuperscript{4}</td>
<td>3.4 ± 1.4</td>
<td>3.1 ± 1.3</td>
<td>3.1 ± 1.3</td>
</tr>
<tr>
<td>Children in household (no.)</td>
<td>1.9 ± 1.0</td>
<td>2.0 ± 1.0</td>
<td>2.2 ± 1.1</td>
<td>2.3 ± 1.1</td>
</tr>
<tr>
<td>Mother’s education (no. of yrs of school completed)</td>
<td>3.7 ± 4.3</td>
<td>3.4 ± 4.2</td>
<td>1.8 ± 3.4</td>
<td>1.5 ± 3.1</td>
</tr>
<tr>
<td>Exclusive breastfeeding before the diarrhea episode (%)\textsuperscript{5}</td>
<td>30.2 (26.4, 34.4)\textsuperscript{2}</td>
<td>24.1 (20.5, 27.9)</td>
<td>38.1 (32.3, 44.2)</td>
<td>10.3 (6.3, 15.6)</td>
</tr>
<tr>
<td>Children with cough or difficulty breathing (%)</td>
<td>34.2 (30.2, 38.4)</td>
<td>30.6 (26.7, 34.7)</td>
<td>30.4 (25.0, 36.2)</td>
<td>28.5 (23.2, 34.3)</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Differences in proportions were tested with Pearson’s chi-square analysis, and differences in means were assessed with Student’s t test.
\textsuperscript{2} Mean; 95% CI in parentheses (all such values).
\textsuperscript{3} Significantly different from placebo group, P < 0.05 (Pearson’s chi-square analysis).
\textsuperscript{4} ± SD (all such values).
\textsuperscript{5} Exclusive breastfeeding was defined as any infant who received only breast milk (no water) during the week before the diarrhea episode.
age. We previously reported that there was no effect of zinc supplementation for the treatment of diarrhea in this population of infants (20). Here we present the longer-term effects of zinc supplementation on infant morbidity and growth.

In our study, 14 d of zinc for the treatment of diarrhea in young infants did not decrease the incidence or prevalence of diarrhea episodes in the 8 wk after treatment. These results differ from the previously reported pooled analysis of short-course supplementation trials conducted in older children, which reported a 34% decrease in the prevalence of diarrhea among zinc-supplemented children when compared with those receiving placebo (10). It is difficult to explain why we observed a 20% increase in the prevalence of diarrhea in the zinc-supplemented infants; there is little biological plausibility for this. Our study observed no significant difference in the incidence of respiratory infections or pneumonia between the groups. Although a statistically significant positive effect of zinc on the incidence of pneumonia has only been observed in longer-duration supplementation trials, a positive trend has been observed among zinc-supplemented children in short-course trials as well (10).

TABLE 2
Incidence and prevalence of diarrhea by supplementation group

<table>
<thead>
<tr>
<th></th>
<th>Zinc (n = 538)</th>
<th>Placebo (n = 536)</th>
<th>P $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of infants with ≥1 episode of any diarrhea (%)</td>
<td>59.5 (55.2, 64.7 $^2$)</td>
<td>58.8 (54.5, 63.0)</td>
<td>0.841</td>
</tr>
<tr>
<td>Proportion of infants with ≥2 episodes of any diarrhea (%)</td>
<td>29.0 (25.2, 33.0)</td>
<td>36.4 (26.5, 34.5)</td>
<td>0.746</td>
</tr>
<tr>
<td>Proportion of infants with ≥1 episode of dysentery (%)</td>
<td>3.5 (2.1, 5.5)</td>
<td>1.7 (0.1, 3.2)</td>
<td>0.049</td>
</tr>
<tr>
<td>Incidence of diarrhea (episodes/mo)</td>
<td>0.618 ± 0.683 $^4$</td>
<td>0.607 ± 0.697</td>
<td>0.810</td>
</tr>
<tr>
<td>Prevalence of diarrhea (d/mo)</td>
<td>2.68 ± 4.11</td>
<td>2.20 ± 3.19</td>
<td>0.030</td>
</tr>
</tbody>
</table>

$^1$ Differences in proportions were assessed by a logistic regression analysis after adjustment for original diarrhea episode lasting >7 d, exclusive breastfeeding upon enrollment, and weight-for-length $z$ score at start of follow-up. Differences in rates were assessed by a robust Poisson regression after adjustment for original diarrhea episode lasting >7 d, exclusive breastfeeding, weight-for-length $z$ score at start of follow-up, age, and sex.

$^2$ $\bar{x}$ ± 95% CI in parentheses (all such values).

$^3$ A new episode of diarrhea is defined as any day of diarrhea with ≥3 diarrhea-free days since the last diarrhea day of the last episode.

$^4$ Dysentery is defined as any day with blood in the stool.

$^5$ $\bar{x}$ ± SD (all such values).
Prevalence of pneumonia (weekly episodes/mo) 0.07/L50512

Grunting, congestion, runny nose, or stuffed nose reported during the previous week

Breastfeeding upon enrollment, and weight-for-length adjustment for original diarrhea episode lasting

Conducted in older children because they had not yet become zinc deficient. Nearly all these young infants were receiving breastmilk, which contains highly bioavailable zinc (32). Osendarp et al (29) supplemented Bangladeshi infants daily from 4–24 wk of age with 5 mg Zn or placebo and did not observe an effect on the incidence or prevalence of diarrhea or on the overall incidence of acute lower respiratory infections. However, when only zinc-deficient infants were assessed, there was a 70% decrease in the incidence of acute lower respiratory infections among infants who were supplemented with zinc compared with those who received placebo. A limitation of the current study was that we did not assess zinc status via serum zinc concentrations or the quantification of dietary zinc intake via breastmilk and complementary foods. For these reasons, we could not assess the variation in effect, if any, by baseline zinc status.

The exact mechanism by which zinc is an effective therapy for diarrhea and also prevents subsequent morbidity is not clearly known. One plausible mechanism is via the important role zinc plays in maintaining proper immune function and specifically enhancing cellular immunity (33). During the first year of life, the cellular immune system is still developing, thus the young infant may not respond to zinc in the same way as an older infant.

Further investigation of the exact mechanisms by which zinc helps maintain immune function and prevents infectious diseases is needed to clarify why the positive effect of zinc is observed in older infants, but not in all infants under 6 mo of age.

Additionally, no significant observed effect of zinc on growth was observed during the 2 mo after the diarrhea episode in these young infants. Of the 4 trials giving zinc for longer periods of time in this age group, 2 found limited effects on growth (26–29). One found a positive effect of zinc among infants with low serum zinc at the start of supplementation (29), and the other found a positive effect on weight during segments of the follow-up time but not overall (28). Zinc supplementation can be an important intervention to improve growth where dietary zinc intake among children is low and where high rates of stunting are present (30). In Ethiopia, Umeta et al (31) randomly assigned 100 stunted and 100 nonstunted infants 6–12 mo of age to receive zinc or placebo daily for 6 mo. Zinc supplementation increased the length of infants in both groups, but this was more pronounced among infants who were stunted at baseline. Although 27.8% of our enrolled infants were stunted at baseline, this is likely to reflect small size at birth in this young age group, rather than growth faltering as seen in later infancy. Throughout the follow-up period the infants in our study did not grow at an adequate rate to achieve optimal growth, which resulted in decreasing z scores. We observed greater growth faltering in length than in weight; stunting rates (<−2 LAZ) increased from 27.8% to 31.7% in 8 wk.

It is possible that the infants enrolled in the current study did not respond to the zinc with the benefits observed in other studies conducted in older children because they had not yet become zinc deficient. Nearly all these young infants were receiving breastmilk, which contains highly bioavailable zinc (32). Osendarp et al (29) supplemented Bangladeshi infants daily from 4–24 wk of age with 5 mg Zn or placebo and did not observe an effect on the incidence or prevalence of diarrhea or on the overall incidence of acute lower respiratory infections. However, when only zinc-deficient infants were assessed, there was a 70% decrease in the incidence of acute lower respiratory infections among infants who were supplemented with zinc compared with those who received placebo. A limitation of the current study was that we did not assess zinc status via serum zinc concentrations or the quantification of dietary zinc intake via breastmilk and complementary foods. For these reasons, we could not assess the variation in effect, if any, by baseline zinc status.

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TABLE 3
Prevalence of respiratory illnesses and proportion of infants brought to a health facility by supplementation group

<table>
<thead>
<tr>
<th></th>
<th>Zinc (n = 538)</th>
<th>Placebo (n = 536)</th>
<th>p1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of infants with ≥1 episode of respiratory infection (%)</td>
<td>45.5 (41.3, 49.9)</td>
<td>45.3 (41.1, 49.7)</td>
<td>0.448</td>
</tr>
<tr>
<td>Prevalence of respiratory infections (weekly episodes/mo)</td>
<td>0.681 ± 1.064</td>
<td>0.680 ± 1.027</td>
<td>0.309</td>
</tr>
<tr>
<td>Proportion of infants with pneumonia (%)</td>
<td>11.7 (9.1, 14.7)</td>
<td>9.7 (7.3, 12.5)</td>
<td>0.232</td>
</tr>
<tr>
<td>Prevalence of pneumonia (weekly episodes/mo)</td>
<td>0.07 ± 0.234</td>
<td>0.067 ± 0.242</td>
<td>0.702</td>
</tr>
<tr>
<td>Proportion who sought care at any health care facility (%)</td>
<td>39.4 (35.3, 43.7)</td>
<td>37.3 (33.2, 41.6)</td>
<td>0.239</td>
</tr>
<tr>
<td>Proportion of infants hospitalized (kept over night) at a health care facility (%)</td>
<td>1.9 (0.9, 3.4)</td>
<td>1.1 (0.4, 2.4)</td>
<td>0.148</td>
</tr>
</tbody>
</table>

1 Differences in proportions were assessed by a logistic regression analysis with adjustment for original diarrhea episode lasting >7 d, exclusive breastfeeding upon enrollment, and weight-for-length z score at start of follow-up. Differences in rates were assessed by a robust Poisson regression after adjustment for original diarrhea episode lasting >7 d, exclusive breastfeeding, weight-for-length z score at start of follow-up, age, and sex.

2 Respiratory infection is defined as any of the following symptoms: cough, fast breathing, difficulty breathing, chest indrawing, wheezing, nasal flaring, grunting, congestion, runny nose, or stuffed nose reported during the previous week

3 Mean; 95% CI in parentheses (all such values).

4 ± SD (all such values).

5 Pneumonia is defined as cough and difficult or fast breathing (respiratory rate ≥60 breaths/min for infants aged <2 mo or ≥50 breaths/min for infants aged ≥2 mo).

TABLE 4
Mean weight and length at start, week 4, and week 8 of follow-up

<table>
<thead>
<tr>
<th></th>
<th>Length</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cm</td>
<td>kg</td>
</tr>
<tr>
<td>Group</td>
<td>Start of follow-up</td>
<td>Week 4</td>
</tr>
<tr>
<td>Zinc</td>
<td>58.53 ± 4.54</td>
<td>60.33 ± 4.46</td>
</tr>
<tr>
<td>Placebo</td>
<td>58.66 ± 4.26</td>
<td>60.42 ± 4.22</td>
</tr>
</tbody>
</table>

1 All values are ± SD.
Baseline anthropometric status at the start of the weekly follow-up and mean change 8 wk after the supplementation period by supplementation group.*

<table>
<thead>
<tr>
<th></th>
<th>Zinc (n = 522)</th>
<th>Placebo (n = 513)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start of follow-up</td>
<td>$-1.27 \pm 1.32$</td>
<td>$-1.41 \pm 1.28$</td>
</tr>
<tr>
<td>Change</td>
<td>$0.11 \pm 0.52$</td>
<td>$0.12 \pm 0.60$</td>
</tr>
<tr>
<td>LAZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start of follow-up</td>
<td>$-1.29 \pm 1.28$</td>
<td>$-1.29 \pm 1.35$</td>
</tr>
<tr>
<td>Change</td>
<td>$-0.19 \pm 0.63$</td>
<td>$-0.19 \pm 0.65$</td>
</tr>
<tr>
<td>WLZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start of follow-up</td>
<td>$-0.31 \pm 1.54$</td>
<td>$-0.51 \pm 1.40$</td>
</tr>
<tr>
<td>Change</td>
<td>$0.25 \pm 0.94$</td>
<td>$0.25 \pm 0.97$</td>
</tr>
</tbody>
</table>

* All values are $\bar{x} \pm SD$. WAZ, weight-for-age $z$; LAZ, length-for-age $z$; WLZ, weight-for-length $z$. Differences between zinc and placebo were assessed by using ANOVA with control for corresponding $z$ score at start of follow-up, exclusive breastfeeding status, and total days of diarrhea during follow-up. No statistically significant differences were observed between the supplementation groups.

Because we previously reported no effect of zinc on the duration and severity of the diarrhea episode (20), it is not surprising that here we report no positive effect on morbidity or growth during the 8 wk of follow-up in the current study population. The only other study of zinc for diarrhea treatment in this age group also observed no effect of zinc on diarrhea duration, but had no follow-up assessment after recovery from illness (34). Additional research among specific subgroups of infants who appear to benefit from zinc supplementation, such as low-birth-weight and small-for-gestational-age infants, may help clarify current uncertainties and guide targeted zinc interventions.

The authors thank Olivier Fontaine for technical support and the World Health Organization for providing the zinc and placebo dispersible tablets for this trial. The authors also thank Dilip Mahalanabis for technical support and Kiran Bhatia for data management skill, both of whom are from the Society for Applied Studies, India.

CLFW, REB, ZAB, and NB conceptualized the study. CLFW, ZAB, NB, TT, FS, and ST enrolled patients, implemented the study protocol, and provided ongoing monitoring. CLFW analyzed the data. CLFW wrote the manuscript. CLFW, REB, ZAB, and NB were responsible for editing the final manuscript. The expanded Zinc Study Group was also part of data collection, data management, and technical support for the duration of the study. The Zinc Study Group includes Shahid Rasool (Aga Khan University), S Qamaruddin Nizami (Aga Khan University), Tsemunaro Rongsen (Society for Applied Studies), Vandna Suri (Society for Applied Studies), Silesi Luloged (Addis Ababa University), and Yeshawetsayeh Tesfaye (Addis Ababa University). The authors have no personal or financial conflicts of interests.

REFERENCES

13. Roy SK, Tomkins AM, Mahalanabis D, et al. Impact of zinc supplementa-
15. Mbonye AK. Prevalence of childhood illnesses and care-seeking prac-
23. WHO Multicenter Growth Reference Study Group. WHO child growth standards: length/height-for-age, weight-for-age, weight-for-length, weight-for-height, and body mass index-for-age: methods and develop-
25. Baqui AH, Black RE, El Arifeen S, et al. Effect of zinc supplementation started during diarrhoea on morbidity and mortality in Bangladeshi chil-
Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women1–4

Pamela J Mink, Carolyn G Scrafford, Leila M Barraj, Lisa Harnack, Ching-Ping Hong, Jennifer A Nettleton, and David R Jacobs Jr

ABSTRACT

Background: Dietary flavonoids may have beneficial cardiovascular effects in human populations, but epidemiologic study results have not been conclusive.

Objective: We used flavonoid food composition data from 3 recently available US Department of Agriculture databases to improve estimates of dietary flavonoid intake and to evaluate the association between flavonoid intake and cardiovascular disease (CVD) mortality.

Design: Study participants were 34 489 postmenopausal women in the Iowa Women’s Health Study who were free of CVD and had complete food-frequency questionnaire information at baseline. Intakes of total flavonoids and 7 subclasses were categorized into quintiles, and food sources were grouped into frequency categories. Proportional hazards rate ratios (RR) were computed for CVD, coronary heart disease (CHD), stroke, and total mortality after 16 y of follow-up.

Results: After multivariate adjustment, significant inverse associations were observed between anthocyanidins and CHD, CVD, and total mortality [RR (95% CI) for any versus no intake: 0.88 (0.78, 0.99), 0.91 (0.83, 0.99), and 0.90 (0.86, 0.95)]; between flavanones and CHD [RR for highest quintile versus lowest: 0.78 (0.65, 0.94)]; and between flavones and total mortality [RR for highest quintile versus lowest: 0.88 (0.82, 0.96)]. No association was found between flavonoid intake and stroke mortality. Individual flavonoid-rich foods associated with significant mortality reduction included bran (added to foods; associated with stroke and CVD); apples or pears or both and red wine (associated with CHD and CVD); grapefruit (associated with CHD); strawberries (associated with CVD); and chocolate (associated with CVD).

Conclusion: Dietary intakes of flavanones, anthocyanidins, and certain foods rich in flavonoids were associated with reduced risk of death due to CHD, CVD, and all causes. Am J Clin Nutr 2007;85:895–909.

KEY WORDS Flavonoids, diet, coronary heart disease, cardiovascular disease, mortality, postmenopausal women, prospective studies

INTRODUCTION

Flavonoids are polyphenolic compounds found in small quantities in numerous plant foods, including fruit and vegetables, tea, wine, nuts and seeds, and herbs and spices (1, 2). Flavonoids are antioxidants and thus may reduce the oxidation of LDL cholesterol, which is thought to be involved in the development of atherosclerotic diseases (3–5). Other hypothesized mechanisms by which flavonoids may have cardioprotective effects include antinflammatory action, improvement in endothelial function, and inhibition of platelet aggregation (3, 6, 7).

Epidemiologic data suggest that dietary flavonoids may have beneficial cardiovascular effects in human populations. Several prospective studies have reported statistically significant inverse associations between total flavonoid intake or the intake of specific classes of flavonoids and cardiovascular disease (CVD) incidence or mortality (2, 8–14), whereas other prospective studies have not (15–17). Epidemiologic studies of flavonoid intake and stroke incidence or mortality have also been inconsistent (9, 10, 18, 19). Sagara et al (20) reported data from an intervention study indicating that isoflavones may reduce baseline measures of several CVD risk factors, including systolic and diastolic blood pressures, total cholesterol, and non-HDL cholesterol.

Most epidemiologic studies to date have been limited by the information available in nutrient databases, and they have focused primarily on flavonoids (quercetin, kaempferol, and myricetin), flavones (luteolin and apigenin), catechins (flavan-3-ols), and isoflavones. In 2003 and 2004, the US Department of Agriculture (USDA) released new databases of flavonoid (flavonols, flavanones, flavan-3-ols, anthocyanidins) and proanthocyanidin content of selected foods (225 and 205 foods, respectively) (21, 22). A database of isoflavone concentrations in selected (128) foods has been available since 1999 (23). These databases contain the most recent publicly available data on flavonoid content of foods, reported as aglycones, and include additional data generated by the USDA Agricultural Research

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2 The opinions expressed herein are those of the authors and do not necessarily represent those of International Life Sciences Institute (ILSI) North America. ILSI North America’s programs are supported primarily by its industry membership.

3 Supported by the Flavonoids Project Committee of the North American branch of the International Life Sciences Institute. The Iowa Women’s Health Study was funded by grant no. RO1 CA39742 from the National Cancer Institute.

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Service. The combination of these 3 databases provides a more complete picture of the flavonoid concentrations in foods than was found with previously available databases.

The purpose of the current study was to examine the association between flavonoid intake and CVD and stroke in a prospective cohort study of postmenopausal women by using newly available nutrient composition information to calculate the intake of flavonoids. Our objective was to evaluate the hypothesis that flavonoid intake is inversely associated with CVD mortality. In addition, we evaluated the relation between individual foods that are major sources of flavonoids or that have high flavonoid content and specific mortality endpoints.

SUBJECTS AND METHODS

Study population

Participants in the Iowa Women’s Health Study (IWHS) were recruited from a random sample of 99,826 women aged 55–69 y who had a valid Iowa driver’s license in 1985. A 16-page baseline questionnaire was mailed to these women in 1986. The 41,836 women who returned the survey make up the cohort under study. Characteristics of nonrespondents were reported elsewhere (24). The baseline questionnaire included a food-frequency questionnaire (FFQ) and other questions relevant to the risk of coronary heart disease (CHD) and stroke (10). Follow-up questionnaires to confirm participants’ place of residence, vital status, and ascertain additional information were mailed in 1987, 1989, 1992, and 1997.

Dietary assessment

The 127-item FFQ was adapted, with minor modifications, from the questionnaire used in the 1984 Nurses’ Health Study survey (25). The FFQ, part of the 1986 baseline questionnaire, had detailed information on fruit (15 items) and vegetables (29 items) and included information on individual foods with high flavonoid content (eg, tea, chocolate, red wine, blueberries, and strawberries). For each food, a portion size was specified, and participants were asked to choose 1 of 9 frequency categories ranging from “as in the questionnaire” “never or less than once per month” to “6+ day.” Onions, which have been cited in the literature as a potential significant source of flavonoids, were not included on the questionnaire. Participants were asked to indicate foods (serving size and servings/wk) that they usually ate ≥1 time/wk but that were not listed on the questionnaire. The brand and type of cold breakfast cereal usually consumed were ascertained; the questionnaire also collected information on the use of vitamin supplements. Validity of the FFQ to ascertain nutrient intake was evaluated in a subgroup of 44 women by comparing the mean nutrient intake according to the questionnaire with that estimated from five 24-h dietary recall interviews (26). The correlation coefficients for alcohol, caffeine, and vitamin C (without supplements) were 0.32, 0.82, and 0.53, respectively. The FFQ was not evaluated for its ability to assess flavonoid intake in this population; however, the questionnaire was previously validated in another population (27). Feskanich et al (27) reported correlation coefficients of 0.70, 0.77, and 0.83 for the important sources of flavonoids apples, tea, and red wine, respectively.

Follow-up

Women were followed annually through the State Health Registry of Iowa, which collects information on deaths in Iowa. Deaths were also identified through the 4 follow-up questionnaires by linking women who did not respond to the questionnaire with the National Death Index. We used the International Classification of Diseases, 9th Revision (28) to classify the reported cause of death in the following categories (not mutually exclusive): CHD (codes 410–414 (ischemic heart disease) or code 429.2 (arteriosclerotic heart disease)); stroke (codes 430–438); total CVD (codes 390–459); and total mortality (all ICD-9 codes for mortality). Follow-up time for each woman was calculated as the number of days from the date of return of the baseline questionnaire to the date of death or 31 December 2002, whichever came first.

Each woman’s return of the questionnaire was considered to provide consent. This study was approved by the Committee on the Use of Human Subjects in Research of the University of Minnesota.

Data analysis

Cohort for analysis

Women were excluded from the current analyses if they reported on the baseline questionnaire that they were premenopausal (n = 569); that they had been told by a doctor that they had heart disease or angina or had had a heart attack (n = 4115); if their FFQ was incomplete (ie, ≥30 food items were left blank) (n = 2782); or if their total energy intake was implausibly low (<600 kcal/d) or high (≥5000 kcal/d) (n = 538). Numbers excluded are not mutually exclusive. In addition, 3 women who had zero total person-years were excluded. After these exclusions, 34,489 women remained eligible for follow-up. In these women, 7,091 total deaths, 2,316 CVD deaths, 1,329 CHD deaths, and 469 deaths due to stroke occurred.

Dietary variables

Preparing the dietary variables for analysis involved 2 main steps: 1) deriving estimates of the flavonoid content in foods on the IWHS FFQ on the basis of data from the 3 USDA databases and 2) calculating estimated daily flavonoid intake for each participant. We first merged the 3 USDA databases—the isoflavone database (23), the flavonoid database (21), and the proanthocyanidin database (22). The compounds included in each database, along with typical high-content foods, are summarized in Table 1. Merging of the databases resulted in a single datafile, which included the flavonoid contents reported in any of the 3 databases for each 5-digit USDA food code reported. If a food was included in both the flavonoid and proanthocyanidin databases, and if both a flavan-3-ol value (flavonoid database) and a monomer value (proanthocyanidin database) were reported, we averaged the 2 values. Several flavan-3-ols (eg, theaflavins and thearubigins) are found only in tea, and they were not included in the proanthocyanidin database; therefore, we did not average the 2 values and instead used the flavan-3-ol value for tea.

For each food, we computed the sum of assessed flavonoids (total flavonoids) for each flavonoid subclass by summing the flavonoid values for the matching food in the USDA databases and the flavonoid content of the matching USDA foods. For items on the FFQ that included just one food (eg, bananas), the flavonoid value for the matching food in the USDA databases

Table 1. Merging of the databases resulted in a single datafile, which included the flavonoid contents reported in any of the 3 databases for each 5-digit USDA food code reported. If a food was included in both the flavonoid and proanthocyanidin databases, and if both a flavan-3-ol value (flavonoid database) and a monomer value (proanthocyanidin database) were reported, we averaged the 2 values. Several flavan-3-ols (eg, theaflavins and thearubigins) are found only in tea, and they were not included in the proanthocyanidin database; therefore, we did not average the 2 values and instead used the flavan-3-ol value for tea.

For each food, we computed the sum of assessed flavonoids (total flavonoids) for each flavonoid subclass by summing the appropriate compounds (Table 1). For each item on the FFQ (127 items) or each item written in by a participant as a food usually eaten ≥1 time/wk (105 foods), we attempted to identify matching food(s) in the USDA databases. We then assigned these FFQ items the flavonoid content of the matching USDA foods. For items on the FFQ that included just one food (eg, bananas), the flavonoid value for the matching food in the USDA databases
was used. For items in the IWHS FFQ that included >1 food (eg, yams or sweet potatoes), we calculated a weighted average of flavonoid values for corresponding items in the USDA databases, in which the weights for each food were based on the per capita consumption amount for that food as reported in the USDA’s Economic Research Service Food Availability Database for 1986 (the year the FFQ was administered to the IWHS participants) (29). If no data were available, we used per capita consumption estimates from USDA’s 1994–1996 and 1998 Continuing Survey of Food Intakes by Individuals (CSFII) (30). Items on the questionnaire that were mixed dishes (eg, pizza) or that included a combination of foods (eg, mixed vegetables) were assigned a weighted value on the basis of a USDA standard recipe. In the conduct of the matching, some instances occurred in which data were available but the food form or preparation method did not match (eg, dried rather than fresh apricots). When this occurred, we calculated a default processing factor from available flavonoid concentration data for similar foods. Foods in the IWHS FFQ that were not in any of the USDA flavonoid databases were assumed to contain no flavonoids.

We calculated total weekly consumption of a given flavonoid class by using the following equation:

\[ \text{FL}_{\text{intake}} = \sum_{i} \text{FL}_{\text{cont}} \times \text{food}_{\text{cons}} \]  

where \( \text{FL}_{\text{intake}} \) is the weekly intake (mg/wk) of flavonoid class \( (j) \) by the participant \( (i) \), \( \text{FL}_{\text{cont}} \) is the flavonoid class \( (j) \) amount (mg) in one serving of food \( (k) \), and \( \text{food}_{\text{cons}} \) is the times per week that a food was reported to have been consumed \( (k) \) by the participant \( (i) \). Daily intake amounts were then derived by dividing the weekly amounts by 7.

Because the USDA databases have not been used in other epidemiologic studies to date, we compared correlation coefficients comparing for intakes of flavonoids that were evaluated in previous studies (31), and we calculated correlations among flavonoids evaluated in this study, as well as correlations between values obtained in this study compared with intakes calculated by Sampson et al (31).

### Statistical analysis

We created quintiles of dietary flavonoid intakes and calculated the median intake and range of intakes for total flavonoids and each flavonoid subclass. We summarized the baseline characteristics of the cohort (potential confounding factors) and stratified by quintile of total flavonoid intake. Mean values of the dietary variables were adjusted for total energy intake by using the residual method (32).

We estimated rate ratios (RR) associated with quintiles of flavonoid intake by using Cox proportional hazards analyses with the STCOX command in STATA software (version 7.0; Stata Corp, College Station, TX). In the initial analyses (model 1), intakes were adjusted for age and energy (kcal). We evaluated associations in additional multivariable models, adding covariates in groups. Model 2 included adjustment for age, energy, baseline marital status, education level, physical activity, smoking status (never, former, or current), and estrogen replacement therapy use (never, former, or current). Model 3 added baseline body mass index (BMI; in kg/m²), waist-to-hip ratio (WHR), hypertension, and type 2 diabetes mellitus to the variables listed for model 2. Model 4 was adjusted for the variables in model 3, plus intake of the following dietary or nutrient factors: whole grains, fish and seafood, saturated fat, polyunsaturated fat, cholesterol, dietary fiber, vitamin C, vitamin E (from all sources), folate, and β-carotene (from all sources). A final model (model 5 or “parsimonious” model) removed from model 4 variables that had a P value > 0.15. In general, the most parsimonious model included the health and lifestyle variables (eg, marital status, blood pressure, WHR, physical activity, and smoking) but not the dietary variables. A typical exception was the inclusion in the final model of whole-grain intake and, occasionally, polyunsaturated fat intake.

We ran additional analyses by stratifying on baseline smoking status (ever-smoker or never-smoker), and obesity (obese: BMI ≥ 30; not obese: BMI < 30) to informally evaluate potential effect modification by these factors. Interaction terms between flavonoid intakes and each of these factors were also added to models 1, 3, and 4, and likelihood ratio chi-square tests were used to compare the main effects models to the models that included interaction effects and to formally test for statistical interactions. Because diabetic women may be more likely than nondiabetic women to change their diets, we conducted the primary analyses a second time after excluding women with self-reported type 2 diabetes mellitus at baseline (n = 1772) and compared these results with those from the total analytic cohort.
We evaluated the relation between the intake of select individual foods and CVD, CHD, and stroke mortality endpoints by using multivariate models similar to those described above. Individual flavonoid-containing foods were included for analysis if 1) the correlation between food intake and total flavonoid or flavonoid subclass intake was ≥0.5; 2) flavonoid intake from the food contributed to ≥1% of total flavonoid intake in these data; or 3) the food was previously determined, in the scientific literature, to be associated with reduced CVD risks. Selected foods (percentage contribution to total flavonoid intake) included tea (26%), apples and pears (17%), bran added to food (9%), beans or lentils (9%), peaches (5%), oranges (5%), orange juice (5%), strawberries (4%), grapefruit (4%), other fruit juices (3%), chocolate (2%), blueberries (1%), red wine (<1%), grapefruit juice (<1%), grapes and raisins (<1%), apple juice (<1%), apple sauce (<1%), tomatoes (<1%), tomato juice (<1%), broccoli (<1%), celery (<1%), Brussels sprouts (<1%), string beans (<1%), and kale or mustard greens (<1%). Intakes were not divided into quintiles because of the large variability in the number of participants reporting consumption of the selected foods and the skewed distribution of the amounts consumed. Instead, we created categories of food intake—<1 time/wk, 1 time/wk, and >1 time/wk. In a few cases, there were so few consumers of a food that the categories were collapsed to nonconsumers (never or <1 time/mo) and consumers (≥1 times/mo) to allow for a sufficient sample size in each category. Food intake was initially adjusted for age and energy intake; additional multivariable models were run only for those foods that showed a significant association (P < 0.05) with the mortality endpoints.

We tested for evidence of a linear trend by evaluating the intake of total flavonoids and the subclasses (except anthocyanidins, for which the highest category test and the trend test are equivalent) as continuous variables with the quintiles coded to the median value of each quintile in separate proportional hazards regression models. We did not evaluate the dose response for individual foods because of the skewed distribution of intakes in the cohort.

All analyses, including Cox proportional hazards regression analyses, were conducted with the use of SPSS for WINDOWS software (version 7.0; SPSS Institute, Chicago, IL) and STATA software (version 7.0).

RESULTS

Previous reports from this cohort showed that recognized risk factors for CHD, reported at baseline—hypertension, type 2 diabetes mellitus, current smoking, low physical activity, higher BMI, and higher WHR—were associated with higher rates of CHD mortality (10, 33, 34). We calculated correlation coefficients to compare flavonoid intakes calculated by Sampson et al (31) and previously examined in the IWHS (10) with those derived in the current study. The dataset evaluated by Sampson et al included only 3 flavonols (quercetin, kaempferol, and myricetin) and the flavones luteolin and apigenin. Flavonol intakes based on the concentrations from the study by Sampson et al were highly correlated with the corresponding intakes in the current analysis (0.80–0.95) and with intakes of flavan-3-ols and proanthocyanidins derived in the current analysis (0.68–0.89), whereas flavone intakes showed a weaker correlation (0.31–0.47). Intakes of individual flavonoids according to the concentrations determined by Sampson et al (31) were highly intercorrelated (0.77–0.84), whereas the correlation among flavonones was more modest (r = 0.26). In the current analysis, the flavan-3-ol monomers, flavonols, and proanthocyanidins were highly correlated with each other (0.75–0.81). Other variables in the current flavonoid dataset were less correlated.

The distribution of potential risk factors for CVD and stroke mortality according to total flavonoid intake level is shown in Table 2. The upper quintiles of flavonoid intake were associated with older age, lower BMI, lower WHR, greater physical activity, smaller proportions of current smokers, greater proportions of multivitamin users, education beyond high school, and current marriage. In addition, the upper quintiles were associated with greater intakes of whole grains, dietary fiber, vitamin C (without supplements), vitamin E (from any source), folate (from any source), and β-carotene (from any source) and lower intakes of alcohol, saturated fat, and cholesterol. The prevalence of type 2 diabetes mellitus and high blood pressure did not differ significantly across quintiles of total flavonoid intake.

Associations between intake of total flavonoids and flavonoid subclasses and mortality endpoints are shown in Tables 3, 4, 5, and 6. Because the results for models 2–5 did not appear to differ materially, we present only the results from model 1 (age- and energy-adjusted) and model 3 (multivariate-adjusted). After adjustment for age and energy, there was a significant inverse association between total mortality and each upper quintile of total flavonoids and each flavonoid subclass intake (Table 3). These associations approached 1.0 after adjustment for additional covariates, however, and only the associations with anthocyanidins and flavones remained significant in the multivariate models. No association was found between stroke mortality and any of the flavonoid classes; the tests for trend were not significant (Table 4). Intakes of total flavonoids (P for trend = 0.075), anthocyanidins, flavanones, flavonones, and proanthocyanidins were inversely associated with CHD mortality in models after adjustment for age and energy, and anthocyanidins and flavonones remained significantly inversely associated after multivariate adjustment (Table 5). For total CVD mortality, significant inverse associations after adjustment for age and energy intake were observed for intake of total flavonoids, anthocyanidins, flavanones, flavones, and proanthocyanidins (Table 6). After multivariate adjustment, the relative risk for the upper category of anthocyanidins was attenuated but remained significant (P for trend = 0.032). The P values of the tests for trend for intake of flavanones were 0.001 after adjustment for age and energy, and 0.054 for the multivariate-adjusted model. The multivariate-adjusted relative risk in the "parsimonious" model for the highest versus lowest quintile was 0.77 (95% CI: 0.65, 0.92; P for trend = 0.002) (data not shown), whereas the corresponding RR in the multivariate model was 0.88 (0.77, 1.01) (see Table 6).

Most of the associations between the intakes of total flavonoids and subclasses and the mortality endpoints changed (ie, became closer to the null) and were no longer significant after adjustment for nondietary risk factors for CVD (model 3). We evaluated results from a “full” multivariate model (model 4), which included dietary factors associated with CVD. These dietary factors may have been correlated with the flavonoid and food variables, which were the independent variables of primary interest in the model; however, results from the models without these variables did not differ materially from results from the full model. The parsimonious model (model 5) did not include most of the dietary or nutrient variables from model 4, and again,
TABLE 2
Baseline characteristics by quintile (Q) of total flavonoid intake for 34 492 cardiovascular disease–free postmenopausal women (Iowa Women’s Health Study, 1986)\(^1\)

<table>
<thead>
<tr>
<th>Total flavonoids (mg/d)</th>
<th>Q1 ((n = 6898))</th>
<th>Q2 ((n = 6899))</th>
<th>Q3 ((n = 6898))</th>
<th>Q4 ((n = 6899))</th>
<th>Q5 ((n = 6898))</th>
<th>P for trend(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous variables</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline age (y)</td>
<td>61.1 ± 4.1(^1)</td>
<td>61.4 ± 4.2</td>
<td>61.5 ± 4.2</td>
<td>61.8 ± 4.2</td>
<td>61.8 ± 4.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>26.9 ± 5.2</td>
<td>27 ± 5.1</td>
<td>27 ± 5</td>
<td>27 ± 5</td>
<td>26.7 ± 5</td>
<td>0.014</td>
</tr>
<tr>
<td>WHR</td>
<td>0.8429 ± 0.0896</td>
<td>0.8368 ± 0.0844</td>
<td>0.8357 ± 0.0872</td>
<td>0.8349 ± 0.0817</td>
<td>0.8319 ± 0.0846</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Energy intake (kcal/d)</td>
<td>1468.5 ± 481.6</td>
<td>1684.2 ± 511.5</td>
<td>1826.6 ± 553.5</td>
<td>1960.4 ± 584.1</td>
<td>2064 ± 694</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alcohol (g/d)(^4)</td>
<td>5.3 ± 10.8</td>
<td>4.1 ± 9</td>
<td>3.6 ± 8.4</td>
<td>3.2 ± 8</td>
<td>3.1 ± 8.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Whole-grain intake (servings/wk)(^4)</td>
<td>9.5 ± 7.5</td>
<td>10.5 ± 7.7</td>
<td>10.9 ± 7.7</td>
<td>11.7 ± 8.1</td>
<td>14.2 ± 10.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dietary fiber (g/d)(^4)</td>
<td>16.3 ± 4</td>
<td>18.3 ± 4.5</td>
<td>19.8 ± 4.9</td>
<td>21.2 ± 5.2</td>
<td>23.2 ± 7.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Saturated fat (g/d)(^4)</td>
<td>26.4 ± 4.9</td>
<td>24.9 ± 4.8</td>
<td>24.1 ± 5.1</td>
<td>23.2 ± 5.4</td>
<td>22.2 ± 6.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Polyunsaturated fat (g/d)(^4)</td>
<td>12.3 ± 2.9</td>
<td>12.2 ± 3</td>
<td>12.1 ± 3.6</td>
<td>11.9 ± 3.4</td>
<td>11.8 ± 3.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cholesterol (g/d)(^4)</td>
<td>285.6 ± 92.6</td>
<td>277.9 ± 84.2</td>
<td>274.9 ± 91.1</td>
<td>272.7 ± 104</td>
<td>261.2 ± 107</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin C without supplementation (mg/d)(^4)</td>
<td>114.3 ± 48</td>
<td>140.4 ± 53.2</td>
<td>157.5 ± 60.8</td>
<td>175.1 ± 72.1</td>
<td>185.2 ± 98</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total vitamin E activity (IU/d)(^7)</td>
<td>51.6 ± 130.8</td>
<td>61.4 ± 141.8</td>
<td>65.2 ± 143.6</td>
<td>74 ± 154.6</td>
<td>83.8 ± 165.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Folate (µg/d)(^4)</td>
<td>364 ± 210</td>
<td>399.8 ± 215.9</td>
<td>426.1 ± 225.8</td>
<td>453.2 ± 236.7</td>
<td>491.6 ± 258.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Folate without supplementation (µg/d)(^4)</td>
<td>266.4 ± 79.1</td>
<td>292.5 ± 84.2</td>
<td>311.4 ± 95</td>
<td>330.7 ± 105.9</td>
<td>355.5 ± 122.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>β-Carotene (IU/d)(^7)</td>
<td>7339.2 ± 5902.5</td>
<td>8439.1 ± 6148.8</td>
<td>9278.6 ± 6500.7</td>
<td>10554 ± 8442.8</td>
<td>11383 ± 9959.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Categorical variables</td>
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<tr>
<td>Physical activity (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Low</td>
<td>60</td>
<td>51</td>
<td>45</td>
<td>41</td>
<td>40</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Moderate</td>
<td>23</td>
<td>28</td>
<td>29</td>
<td>30</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>17</td>
<td>21</td>
<td>26</td>
<td>29</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Smoking status (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Never</td>
<td>55</td>
<td>64</td>
<td>69</td>
<td>71</td>
<td>69</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Past</td>
<td>20</td>
<td>19</td>
<td>19</td>
<td>18</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>25</td>
<td>16</td>
<td>13</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Pack-years (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>55</td>
<td>65</td>
<td>69</td>
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<td>Aspirin frequency (%)</td>
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<td>&lt;1/wk</td>
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<td>≥6/wk</td>
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<td>19</td>
<td>20</td>
<td>21</td>
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\(^1\) WHR, waist-to-hip ratio.  
\(^2\) Likelihood ratio (chi-square test).  
\(^3\) ± SD (all such values).  
\(^4\) Adjusted for energy intake (kcal/d).
results did not differ materially from those from the other multivariate models.

We found no material differences between results based on the full cohort and results from a subcohort restricted to women who did not report a history of type 2 diabetes mellitus at baseline. In addition, the pattern of results was generally similar for never-smokers and ever-smokers and for obese and nonobese women. An exception to this was in analyses of flavanones, in which

<table>
<thead>
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<th>Flavonoid intake</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
<th>Q5</th>
<th>P for trend¹</th>
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<td>No. of deaths²</td>
<td>1568 (106 568)</td>
<td>1416 (107 544)</td>
<td>1334 (108 183)</td>
<td>1365 (108 045)</td>
<td>1408 (107 736)</td>
<td></td>
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<tr>
<td>Intake (mg/d)</td>
<td>95.8 (0.6–133.2)¹</td>
<td>167.5 (133.2–201.8)</td>
<td>238.9 (201.9–282)</td>
<td>336.5 (282–425.3)</td>
<td>603.3 (425.3–3524.4)</td>
<td></td>
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<tr>
<td>RR (95% CI)</td>
<td>1.00</td>
<td>0.85 (0.79, 0.92)</td>
<td>0.77 (0.72, 0.83)</td>
<td>0.76 (0.71, 0.82)</td>
<td>0.78 (0.72, 0.84)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>0.95 (0.88, 1.02)</td>
<td>0.92 (0.85, 0.99)</td>
<td>0.94 (0.86, 1.01)</td>
<td>0.96 (0.89, 1.04)</td>
<td>0.716</td>
</tr>
</tbody>
</table>

| Anthocyanidin²   | 2648 (182 302) | 4443 (355 773) |    |    |    | <0.001      |
| No. of deaths²   | 0 | 0.2 (0.01–1040) |    |    |    |             |
| Intake (mg/d)    | 7.6 (0–16.1) | 26.6 (16.1–34) | 40.4 (34–49.5) | 59.9 (49.5–72.8) | 93.7 (72.8–703.3) |             |
| RR (95% CI)      | 1.00 | 0.86 (0.81, 0.90) |    |    |    | <0.001      |
|                  | 1.00 | 0.90 (0.86, 0.95) |    |    |    |             |

| Flavanones       |    |    |    |    |    |             |
| No. of deaths²   | 1570 (106 616) | 1476 (107 195) | 1369 (108 126) | 1342 (107 899) | 1334 (108 239) |             |
| Intake (mg/d)    | 0.1 (0–0.2) | 0.3 (0.2–0.3) | 0.4 (0.3–0.5) | 0.8 (0.5–1) | 1.5 (1–42.7) |             |
| RR (95% CI)      | 1.00 | 0.93 (0.86, 0.99) | 0.85 (0.79, 0.91) | 0.82 (0.76, 0.88) | 0.79 (0.74, 0.86) | <0.001      |
|                  | 1.00 | 0.98 (0.91, 1.05) | 0.92 (0.85, 0.99) | 0.90 (0.83, 0.97) | 0.88 (0.82, 0.96) | 0.001        |

| Flavones         |    |    |    |    |    |             |
| No. of deaths²   | 1560 (106 502) | 1360 (108 023) | 1333 (108 274) | 1414 (107 755) | 1424 (107 520) |             |
| Intake (mg/d)    | 4.1 (0.2–5.4) | 6.6 (5.4–7.7) | 8.9 (7.7–10.3) | 12.1 (10.3–14.6) | 21 (14.6–125.4) |             |
| RR (95% CI)      | 1.00 | 0.85 (0.79, 0.91) | 0.81 (0.75, 0.87) | 0.85 (0.79, 0.91) | 0.85 (0.79, 0.92) | 0.019        |
|                  | 1.00 | 0.91 (0.84, 0.98) | 0.90 (0.84, 0.98) | 0.99 (0.92, 1.07) | 0.95 (0.88, 1.03) | 0.946        |

| Flavonols        |    |    |    |    |    |             |
| No. of deaths²   | 1494 (107 286) | 1368 (107 637) | 1434 (107 975) | 1366 (107 830) | 1429 (107 346) |             |
| Intake (mg/d)    | 0.1 (0–0.1) | 0.1 (0.1–0.2) | 0.3 (0.2–0.3) | 0.3 (0.3–0.5) | 0.8 (0.5–107.8) |             |
| RR (95% CI)      | 1.00 | 0.88 (0.81, 0.94) | 0.92 (0.85, 0.99) | 0.86 (0.79, 0.92) | 0.90 (0.84, 0.97) | 0.198        |
|                  | 1.00 | 0.96 (0.89, 1.03) | 1.02 (0.94, 1.10) | 0.99 (0.92, 1.07) | 1.01 (0.93, 1.10) | 0.455        |

| Isoflavones      |    |    |    |    |    |             |
| No. of deaths²   | 1605 (106 389) | 1408 (107 465) | 1299 (108 171) | 1309 (108 677) | 1470 (107 372) |             |
| Intake (mg/d)    | 4.2 (0–6.8) | 10 (6.8–15.1) | 20.4 (15.1–29.4) | 75.7 (29.4–135.7) | 181.6 (135.7–1049.8) |             |
| RR (95% CI)      | 1.00 | 0.83 (0.77, 0.89) | 0.76 (0.70, 0.82) | 0.75 (0.69, 0.81) | 0.85 (0.79, 0.92) | 0.262        |
|                  | 1.00 | 0.93 (0.86, 1.00) | 0.87 (0.81, 0.94) | 0.88 (0.81, 0.95) | 0.98 (0.91, 1.06) | 0.206        |

| Proanthocyanidins|    |    |    |    |    |             |
| No. of deaths²   | 1548 (106 627) | 1443 (107 483) | 1349 (108 006) | 1376 (107 895) | 1375 (108 064) |             |
| Intake (mg/d)    | 61.9 (0–89.5) | 116.8 (89.5–143.9) | 175.2 (143.9–212.3) | 262.9 (212.3–343.2) | 524 (343.2–3225.6) |             |
| RR (95% CI)      | 1.00 | 0.89 (0.83, 0.96) | 0.80 (0.75, 0.87) | 0.79 (0.73, 0.85) | 0.78 (0.72, 0.84) | <0.001      |
|                  | 1.00 | 0.98 (0.91, 1.05) | 0.94 (0.87, 1.01) | 0.96 (0.89, 1.04) | 0.94 (0.87, 1.02) | 0.213        |

¹ Test for trend conducted with the median value for each quintile.
² Person-years of follow-up in parentheses.
³ Median; range in parentheses (all such values).
⁴ Adjusted for age, energy intake, marital status, education, blood pressure, diabetes, BMI, waist-to-hip ratio, physical activity, smoking, and estrogen use.
⁵ Zero intake versus >0.
inverse associations between intake and mortality due to CVD (P for interaction = 0.03 for model 1), CHD (P for interaction = 0.02 and 0.03 for models 1 and 4, respectively), and all causes (P for interaction = 0.0007, 0.002, and 0.0004 for models 1, 3, and 4, respectively) were observed among ever-smokers, but not among never-smokers. In addition, rate ratios for total mortality comparing the highest versus lowest quintiles of flavonols were significantly reduced for ever-smokers, but not among never-smokers.
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TABLE 5
Rate ratios (RR) of coronary heart disease (CHD) mortality by quintile (Q) of flavonoid intake for 34 492 cardiovascular disease–free postmenopausal
women (Iowa Women’s Health Study)
Flavonoid intake
Q1
Total flavonoids
No. of deaths from CHD2
Intake (mg/d)
RR (95% CI)
Age- and energy-adjusted
Multivariate-adjusted4
Anthocyanidins5
No. of deaths from CHD2
Intake (mg/d)
RR (95% CI)
Age- and energy-adjusted
Multivariate-adjusted4
Flavanones
No. of deaths from CHD2
Intake (mg/d)
RR (95% CI)
Age- and energy-adjusted
Multivariate-adjusted4
Flavones
No. of deaths from CHD2
Intake (mg/d)
RR (95% CI)
Age- and energy-adjusted
Multivariate-adjusted4
Flavonols
No. of deaths from CHD2
Intake (mg/d)
RR (95% CI)
Age- and energy-adjusted
Multivariate-adjusted4
Isoflavones
No. of deaths from CHD2
Intake (mg/d)
RR (95% CI)
Age- and energy-adjusted
Multivariate-adjusted4
Flavan-3-ols or monomers
No. of deaths from CHD2
Intake (mg/d)
RR (95% CI)
Age- and energy-adjusted
Multivariate-adjusted4
Proanthocyanidins
No. of deaths from CHD2
Intake (mg/d)
RR (95% CI)
Age- and energy-adjusted
Multivariate-adjusted4

300 (106 568)
95.8 (0.6–133.2)3
1.00
1.00
513 (182 302)
0
1.00
1.00
296 (106 815)
7.6 (0–16.1)
1.00
1.00
269 (106 616)
0.1 (0–0.2)
1.00
1.00
280 (106 502)
4.1 (0.2–5.4)
1.00
1.00
298 (107 286)
0.1 (0–0.1)
1.00
1.00
300 (106 389)
4.2 (0–6.8)
1.00
1.00
291 (106 627)
61.9 (0–89.5)
1.00
1.00

Q2

Q3

258 (107 544)
247 (108 183)
167.5 (133.2–201.8) 238.9 (201.9–282)
0.81 (0.69, 0.96)
0.87 (0.73, 1.03)

0.74 (0.63, 0.88)
0.85 (0.71, 1.02)

Q4
249 (108 045)
336.5 (282–425.3)
0.72 (0.60, 0.86)
0.85 (0.70, 1.02)

Q5

P for
trend1

275 (107 736)
603.3 (425.3–3524.4)
0.79 (0.66, 0.94)
0.94 (0.78, 1.13)

0.075
0.980

816 (355 773)
0.2 (0.01–1040)
쏝0.001
0.031

0.81 (0.73, 0.91)
0.88 (0.78, 0.99)
282 (107 140)
26.6 (16.1–34)

246 (107 991)
40.4 (34–49.5)

255 (108 502)
59.9 (49.5–72.8)

250 (107 627)
93.7 (72.8–703.3)

0.90 (0.76, 1.06)
0.91 (0.77, 1.08)

0.77 (0.65, 0.91)
0.83 (0.70, 0.99)

0.77 (0.65, 0.91)
0.86 (0.72, 1.02)

0.74 (0.63, 0.88)
0.78 (0.65, 0.94)

284 (107 195)
0.3 (0.2–0.3)

271 (108 126)
0.4 (0.3–0.5)

254 (107 899)
0.8 (0.5–1)

251 (108 239)
1.5 (1–42.7)

1.04 (0.88, 1.23)
1.10 (0.92, 1.31)

0.99 (0.84, 1.18)
1.10 (0.92, 1.31)

0.91 (0.77, 1.09)
0.98 (0.82, 1.18)

0.88 (0.74, 1.05)
0.95 (0.78, 1.14)

267 (108 023)
6.6 (5.4–7.7)

233 (108 274)
8.9 (7.7–10.3)

274 (107 755)
12.1 (10.3–14.6)

275 (107 520)
21 (14.6–125.4)

0.94 (0.79, 1.11)
0.95 (0.80, 1.13)

0.80 (0.67, 0.95)
0.85 (0.71, 1.02)

0.93 (0.78, 1.11)
1.03 (0.86, 1.23)

0.94 (0.79, 1.13)
0.95 (0.79, 1.14)

238 (107 637)
0.1 (0.1–0.2)

265 (107 975)
0.3 (0.2–0.3)

250 (107 830)
0.3 (0.3–0.5)

278 (107 346)
0.8 (0.5–107.8)

0.76 (0.64, 0.90)
0.84 (0.70, 1.00)

0.85 (0.72, 1.01)
0.95 (0.80, 1.13)

0.79 (0.66, 0.94)
0.94 (0.78, 1.12)

0.89 (0.75, 1.06)
1.00 (0.83, 1.19)

233 (107 465)
10 (6.8–15.1)

252 (108 171)
20.4 (15.1–29.4)

253 (108 677)
75.7 (29.4–135.7)

0.74 (0.62, 0.88)
0.82 (0.69, 0.99)

0.79 (0.67, 0.94)
0.89 (0.74, 1.06)

0.78 (0.65, 0.92)
0.91 (0.76, 1.09)

264 (107 483)
116.8 (89.5–143.9)
0.87 (0.74, 1.03)
0.94 (0.79, 1.12)

249 (108 006)
268 (107 895)
175.2 (143.9–212.3) 262.9 (212.3–343.2)
0.79 (0.67, 0.94)
0.91 (0.76, 1.09)

0.81 (0.68, 0.97)
0.94 (0.79, 1.13)

쏝0.001
0.010

0.047
0.147

0.937
0.965

0.995
0.425

291 (107 372)
181.6 (135.7–1049.8)
0.91 (0.77, 1.07)
1.02 (0.86, 1.21)

0.400
0.113

257 (108 064)
524 (343.2–3225.6)
0.77 (0.65, 0.92)
0.91 (0.76, 1.10)

0.021
0.489

1

Test for trend conducted with the median value for each quintile.
Person-years of follow-up in parentheses.
3
Median; range in parentheses (all such values).
4
Adjusted for age, energy intake, marital status, education, blood pressure, diabetes, BMI, waist-to-hip ratio, physical activity, smoking, and estrogen use.
5
Zero intake versus 쏜0.
2

for never-smokers (P for interaction ҃ 0.003, 0.002, and
0.002 for models 1, 3, and 4, respectively).
Consumption of the following foods or beverages from the
FFQ was inversely associated with stroke mortality after adjustment for age and total energy intake (P for trend: 쏝0.001–

0.021): apples and pears, red wine, bran (added to food), and
chocolate (Table 7). Only intake of bran (added to food)
remained statistically significant after multivariate adjustment (P for trend ҃ 0.013). In the analyses of CHD mortality
and foods (Table 8), the age- and energy-adjusted relative


risks were significantly decreased ($P$ for trend: <0.001–0.033) in women reporting consumption of apples and pears, oranges, grapefruit, blueberries, red wine, celery, strawberries, Brussels sprouts, bran (added to food), chocolate, and other fruit juices. Apples and pears, grapefruit, and red wine remained significantly inversely associated with CHD mortality in the multivariate-adjusted and parsimonious models. With the exception of broccoli ($P$ for trend = 0.065) and tomatoes, all of the food...
or beverage items shown in Table 9 were significantly and inversely associated with total CVD mortality after adjustment for age and energy intake. Apples and pears, red wine, strawberries, bran (added to food), and chocolate (P for trend = 0.062) remained significantly associated with a reduced risk of CVD death in the multivariate models. We did not evaluate dose-response patterns for many of the food variables because of the skewed distribution of intake and variable number of consumers in the cohort.

**DISCUSSION**

This prospective study of postmenopausal women, with 16 y of follow-up, is, to our knowledge, the first study that has reported on total flavonoids and on 7 subclasses of flavonoids. We found that dietary intakes of flavanones and anthocyanidins were associated with a decreased risk of death due to CHD, CVD, and all causes combined after multivariate adjustment. We found no association between the intake of total flavonoids or any of the subclasses and stroke mortality. In the analyses of foods, apple and pear and red wine intakes were associated with reduced CHD and total CVD mortality. Grapefruit, a major source of flavonoids, was associated with a lower risk of CHD mortality.

The IWHS previously reported decreased risk for mortality due to CHD but not stroke with greater flavonol and flavone (and broccoli) intakes (10). In a subsequent analysis of this cohort, inverse associations were observed between CHD mortality and intakes of catechins and epicatechins (flavan-3-ols), apples, and wine (11). In contrast with the earlier report (10), in the current study we did not observe a significant inverse association between broccoli intake and CHD mortality. Our findings of decreased CHD and CVD mortality associated with increased intake of apples and pears and red wine are consistent with previous reports from the cohort in the current study (10, 11) and other studies (8, 9, 12, 16), although these associations were sometimes weak. Rimm et al (17) found no association between apple consumption and CHD mortality.

Several studies have reported decreased CHD mortality associated with increased intake of flavonols or one of its major sources, tea (or both) (12, 14, 35). We did not observe a significant reduction in risk of CHD mortality with the intake of tea or

<table>
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<th>Category of food intake</th>
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<th>3</th>
<th>P for trend²</th>
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<td>Apples and pears</td>
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<tr>
<td>No. of deaths from stroke³</td>
<td>141 (133 008)</td>
<td>86 (107 446)</td>
<td>242 (297 621)</td>
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<td>Servings (no./wk)</td>
<td>&lt;1.00</td>
<td>1.00</td>
<td>&gt;1.00</td>
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<tr>
<td>RR (95% CI)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Age- and energy-adjusted</td>
<td>1.00</td>
<td>0.74 (0.57, 0.97)</td>
<td>0.73 (0.59, 0.90)</td>
<td>0.018</td>
</tr>
<tr>
<td>Multivariate-adjusted⁴</td>
<td>1.00</td>
<td>0.85 (0.64, 1.12)</td>
<td>0.85 (0.68, 1.07)</td>
<td>0.284</td>
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<td>Orange juice</td>
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<tr>
<td>No. of deaths from stroke³</td>
<td>192 (209 930)</td>
<td>39 (61 583)</td>
<td>238 (266 562)</td>
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</tr>
<tr>
<td>Servings (no./wk)</td>
<td>&lt;1.00</td>
<td>1.00</td>
<td>&gt;1.00</td>
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<tr>
<td>RR (95% CI)</td>
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<tr>
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<td>0.93 (0.77, 1.13)</td>
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<td>Red wine</td>
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<td>No. of deaths from stroke³</td>
<td>391 (419 239)</td>
<td>78 (118 835)</td>
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<td>Servings (no./wk)</td>
<td>0</td>
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<td>RR (95% CI)</td>
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<td>RR (95% CI)</td>
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<td>Age- and energy-adjusted</td>
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<td>0.61 (0.47, 0.79)</td>
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<td>No. of deaths from stroke³</td>
<td>251 (248 687)</td>
<td>218 (289 388)</td>
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<td>0</td>
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<td>RR (95% CI)</td>
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<td>1.00</td>
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¹ Categories of food intake: 1, <1 time/wk; 2, 1 time/wk; and 3, >1 time/wk.
² Test for trend conducted with the median value for each quintile.
³ Person-years of follow-up in parentheses.
⁴ Adjusted for age, energy intake, marital status, education, blood pressure, diabetes, BMI, waist-to-hip ratio, physical activity, smoking, and estrogen use.
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<th>Category of food intake</th>
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<td>392 (133 008)</td>
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<td>679 (297 621)</td>
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<td>1.00</td>
<td>(&gt;1.00)</td>
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<tr>
<td>RR (95% CI)</td>
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<td>0.80 (0.68, 0.94)</td>
<td>0.74 (0.65, 0.84)</td>
<td>(&lt;0.001)</td>
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<td>439 (191 600)</td>
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<td>(&gt;1.00)</td>
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<tr>
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<td>No. of deaths from CHD</td>
<td>836 (316 726)</td>
<td>195 (86 878)</td>
<td>298 (134 471)</td>
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<td>Servings/wk</td>
<td>(&lt;1.00)</td>
<td>1.00</td>
<td>(&gt;1.00)</td>
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<tr>
<td>RR (95% CI)</td>
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<td>Age- and energy-adjusted</td>
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<td>0.77 (0.67, 0.88)</td>
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<tr>
<td>RR (95% CI)</td>
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<td>No. of deaths from CHD</td>
<td>1119 (419 239)</td>
<td>210 (118 835)</td>
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<tr>
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<td>0</td>
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<td>RR (95% CI)</td>
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<td>0.69 (0.60, 0.80)</td>
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<td>598 (230 778)</td>
<td>293 (119 383)</td>
<td>438 (187 464)</td>
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<td>Servings wk</td>
<td>(&lt;1.00)</td>
<td>1.00</td>
<td>(&gt;1.00)</td>
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<tr>
<td>RR (95% CI)</td>
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<tr>
<td>Age- and energy-adjusted</td>
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<td>0.93 (0.81, 1.07)</td>
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<td>1.02 (0.88, 1.18)</td>
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<td>959 (405 080)</td>
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<tr>
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<td>(&gt;0)</td>
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<tr>
<td>RR (95% CI)</td>
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<td><strong>Brussels sprouts</strong></td>
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<td>425 (161 036)</td>
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<tr>
<td>Servings wk</td>
<td>0</td>
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<tr>
<td>RR (95% CI)</td>
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<tr>
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<td>1.14 (1.02, 1.28)</td>
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<td><strong>Bran (added to food)</strong></td>
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<tr>
<td>No. of deaths from CHD</td>
<td>1112 (436 997)</td>
<td>217 (101 078)</td>
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<tr>
<td>Servings wk</td>
<td>0</td>
<td>(&gt;0)</td>
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<tr>
<td>RR (95% CI)</td>
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<tr>
<td>Age- and energy-adjusted</td>
<td>1.00</td>
<td>0.77 (0.67, 0.89)</td>
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<td>No. of deaths from CHD</td>
<td>667 (248 687)</td>
<td>662 (289 388)</td>
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<td>Servings wk</td>
<td>0</td>
<td>(&gt;0)</td>
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<td>RR (95% CI)</td>
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<td>543 (232 337)</td>
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<td>(&gt;0)</td>
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<td>0.97 (0.86, 1.09)</td>
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</table>

^1 Categories of food intake: 1, \(<1\) time/wk; 2, \(1\) time/wk; 3, \(>1\) time/wk.

^2 Test for trend conducted with the median value for each quintile.

^3 Person-years of follow-up in parentheses.

^4 Adjusted for age, energy intake, marital status, education, blood pressure, diabetes, BMI, waist-to-hip ratio, physical activity, smoking, and estrogen use.
<table>
<thead>
<tr>
<th>Category of food intake</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>P for trend</th>
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<tr>
<td>Apples and pears</td>
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<tr>
<td>No. of deaths from CVD</td>
<td>679 (133 008)</td>
<td>450 (107 446)</td>
<td>1,187 (297 621)</td>
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<td>Servings wk</td>
<td>&lt;1.00</td>
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<td>&gt;1.00</td>
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<tr>
<td>RR (95% CI)</td>
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<tr>
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<td>0.75 (0.68, 0.82)</td>
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<td>No. of deaths from CVD</td>
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<td>&gt;1.00</td>
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<tr>
<td>RR (95% CI)</td>
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<td>No. of deaths from CVD</td>
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<tr>
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<td>RR (95% CI)</td>
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<td>&gt;1.00</td>
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<tr>
<td>RR (95% CI)</td>
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<td>Age- and energy-adjusted</td>
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<td>No. of deaths from CVD</td>
<td>1942 (419 239)</td>
<td>374 (118 835)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Servings/wk</td>
<td>0</td>
<td>&gt;0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age- and energy-adjusted</td>
<td>1.00</td>
<td>0.71 (0.64, 0.80)</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Multivariate-adjusted</td>
<td>1.00</td>
<td>0.80 (0.71, 0.90)</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Celery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of deaths from CVD</td>
<td>1055 (230 778)</td>
<td>504 (119 833)</td>
<td>757 (187 464)</td>
<td></td>
</tr>
<tr>
<td>Servings/wk</td>
<td>&lt;1.00</td>
<td>1.00</td>
<td>&gt;1.00</td>
<td></td>
</tr>
<tr>
<td>RR (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age- and energy-adjusted</td>
<td>1.00</td>
<td>0.91 (0.82, 1.01)</td>
<td>0.85 (0.77, 0.93)</td>
<td>0.002</td>
</tr>
<tr>
<td>Multivariate-adjusted</td>
<td>1.00</td>
<td>0.97 (0.87, 1.08)</td>
<td>0.91 (0.83, 1.01)</td>
<td>0.085</td>
</tr>
<tr>
<td>Tea</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of deaths from CVD</td>
<td>1033 (229 082)</td>
<td>1283 (308 993)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Servings/wk</td>
<td>0</td>
<td>&gt;0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age- and energy-adjusted</td>
<td>1.00</td>
<td>0.90 (0.83, 0.98)</td>
<td></td>
<td>0.011</td>
</tr>
<tr>
<td>Multivariate-adjusted</td>
<td>1.00</td>
<td>0.97 (0.89, 1.06)</td>
<td></td>
<td>0.462</td>
</tr>
<tr>
<td>Grapes and raisins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of deaths from CVD</td>
<td>1701 (386 242)</td>
<td>389 (97 429)</td>
<td>226 (54 404)</td>
<td></td>
</tr>
<tr>
<td>Servings/wk</td>
<td>&lt;1.00</td>
<td>1.00</td>
<td>&gt;1.00</td>
<td></td>
</tr>
<tr>
<td>RR (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age- and energy-adjusted</td>
<td>1.00</td>
<td>0.86 (0.77, 0.97)</td>
<td>0.85 (0.74, 0.98)</td>
<td>0.007</td>
</tr>
<tr>
<td>Multivariate-adjusted</td>
<td>1.00</td>
<td>0.96 (0.85, 1.07)</td>
<td>0.94 (0.81, 1.09)</td>
<td>0.316</td>
</tr>
<tr>
<td>Strawberries</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of deaths from CVD</td>
<td>662 (132 994)</td>
<td>1654 (405 080)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Servings/wk</td>
<td>0</td>
<td>&gt;0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age- and energy-adjusted</td>
<td>1.00</td>
<td>0.82 (0.74, 0.89)</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Multivariate-adjusted</td>
<td>1.00</td>
<td>0.91 (0.82, 1.00)</td>
<td></td>
<td>0.046</td>
</tr>
</tbody>
</table>

(Continued)
flavan-3-ols, nor was the inverse association for these variables and total CVD mortality significant after multivariate adjustment. Two previous studies reported no evidence of a cardioprotective effect of tea (15, 17). A recent report from the Zutphen Elderly Study (36) presented inverse associations between cocoa intake and blood pressure and between CVD and all-cause mortality in men. We observed a borderline significant inverse association between chocolate intake and CVD mortality after multivariate adjustment.

The extent to which our results differed from those of some previous studies may be related to differences in dietary patterns of the cohorts, differences in the foods assessed in the FFQs, differences in the databases used to estimate flavonoid intake, or differences in the cutoffs used to categorize the consumption levels. There were also some internal inconsistencies in our data. For example, patterns of relative risks were not always monotonic. This finding could be due to chance variation or could indicate that the relation between dietary intake of flavonoids and the mortality outcomes investigated is not strictly linear. Significant interactions observed for ever-smokers and never-smokers could represent a biological interaction or could be related to other CVD risk factors that differ between smokers and non-smokers.

Strengths of the current study include its prospective design, large size, and virtually complete follow-up of the cohort for mortality and cause of death. Several limitations of the study and of the USDA databases must, however, be considered. We relied on dietary intake from an FFQ administered at one point in time and did not have updated information. Thus, misclassification of dietary exposure occurs to the extent that women’s diets have changed over the follow-up period. In addition, the potential for a misclassification exists because of misreporting of usual diet. Flavonoid concentrations may be underestimated because of items missing from the questionnaire, such as onions, whole grains, and certain types of berries, which are high in flavonoids. The questionnaire did include an item about blueberry intake. Information on relevant biomarkers of intake, particularly multiple measures over time, would enhance the ability to assess and classify exposure and may also provide insights into mechanisms.

The USDA databases are a compilation of data available in the literature on the flavonoid content of foods. Studies that did not use procedures allowing for good separation of flavonoid compounds were deemed unacceptable by the USDA and were not included in the databases. Nonetheless, the included studies differed with respect to several factors, including overall quality. The USDA assigned each study a rating based on the sampling plan, sample handling, number of samples, analytic method, and analytic quality; however, we did not exclude any of the data in the 3 USDA databases. The published studies had limited data from the United States, often were based on single samples, and often focused on single compounds. Because food and beverage preparation practices vary across countries, comparison across studies may not be possible. This is particularly true for tea, because brewing time practices, which affect the flavonoid content, vary across countries (21). An updated and expanded USDA flavonoid content database, including data from nationally representative US samples of 59 fruit, vegetables, and nuts, was released in January 2007 (37), when this manuscript was in press. Isoflavones and proanthocyanidins were not updated in this latest database.

The flavonoid databases provide limited data from which to understand the effect that processing (eg, drying or baking) has on the flavonoid concentration in foods, expressed as the ratio of flavonoid content in a processed food to that in the unprocessed food. In some cases, when the flavonoid content of the unprocessed food was unavailable, we calculated default processing factors by using those foods that had, for example, flavonoid data

### Table 9 (Continued)

<table>
<thead>
<tr>
<th>Category of food intake</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>( P ) for trend&lt;br&gt;( ^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomatoes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of deaths from CVD&lt;sup&gt;3&lt;/sup&gt;</td>
<td>700 (148 088)</td>
<td>744 (192 919)</td>
<td>872 (197 068)</td>
<td></td>
</tr>
<tr>
<td>Servings/wk</td>
<td>&lt;1.00</td>
<td>1.00</td>
<td>&gt;1.00</td>
<td></td>
</tr>
<tr>
<td>RR (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age- and energy-adjusted</td>
<td>1.00</td>
<td>0.82 (0.74, 0.91)</td>
<td>0.94 (0.85, 1.04)</td>
<td>0.649</td>
</tr>
<tr>
<td>Multivariate-adjusted&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.00</td>
<td>0.83 (0.74, 0.92)</td>
<td>0.93 (0.83, 1.03)</td>
<td>0.998</td>
</tr>
<tr>
<td>Bran (added to food)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of deaths from CVD&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1952 (436 997)</td>
<td>364 (101 078)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Servings/wk</td>
<td>0</td>
<td>&gt;0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age- and energy-adjusted</td>
<td>1.00</td>
<td>0.74 (0.66, 0.82)</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Multivariate-adjusted&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.00</td>
<td>0.86 (0.76, 0.97)</td>
<td></td>
<td>0.014</td>
</tr>
<tr>
<td>Chocolate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of deaths from CVD&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1198 (248 687)</td>
<td>1118 (289 388)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Servings/wk</td>
<td>0</td>
<td>&gt;0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age- and energy-adjusted</td>
<td>1.00</td>
<td>0.83 (0.77, 0.91)</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Multivariate-adjusted&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.00</td>
<td>0.92 (0.84, 1.00)</td>
<td></td>
<td>0.062</td>
</tr>
</tbody>
</table>

<sup>1</sup> Categories of food intake: 1, <1 time/wk; 2, 1 time/wk; 3, >1 time/wk.
<sup>2</sup> Test for trend conducted with the median value for each quintile.
<sup>3</sup> Person-years of follow-up in parentheses.
<sup>4</sup> Adjusted for age, energy intake, marital status, education, blood pressure, diabetes, BMI, waist-to-hip ratio, physical activity, smoking, and estrogen use.
for the raw form and the dried form. However, we do not know the between-food variability in processing.

It is also important to note that the intake estimates presented in the current study are based on the mean concentration of flavonoids in food and do not take into account the large variability in flavonoid content that is seen in many foods. Nevertheless, people eat various foods from various sources, and mean flavonoid content may be the most appropriate measure in epidemiologic studies.

An important limitation inherent in this type of research is multiple comparisons. Because several types of flavonoids and a variety of food sources exist, and because 4 outcome variables were used, many tests were performed. The primary a priori hypothesis was that each flavonoid would be associated with CVD death, and CHD death and stroke death are considered particular examples of that association. The same hypothesis was evaluated for flavonoid-containing foods; here an important caveat is that, given the varied nature of diet, each food contributes little to the overall diet, relative risks for any one food are expected to be less than (but not much less than) 1.0, and statistical power is likely to be low. Examination of total mortality was based on the assumptions that CVD death would play a major role in that endpoint and that flavonoids also protect against some noncardiovascular diseases. Thus P values in the current study must be viewed with caution; “significance” is most securely taken for observations in this study that are consistent with observations in other studies of diet and disease.

This study contributes important information about the relation between the intakes of total flavonoids and 7 subclasses and CVD mortality endpoints. These results alone cannot be considered conclusive, however, because of limitations of the observational study design and of the dietary intake information. Results from this study suggest that the intake of certain subclasses of flavonoids may be associated with lower CHD and total CVD mortality in postmenopausal women. Furthermore, consumption of some foods that are high in flavonoid content or that are among the main sources of flavonoids in the diet of these study participants may have similar associations. The study of potential cardioprotective effects of the intakes of flavanones and anthocyanidins should be replicated in other large prospective studies with comprehensive information about the dietary intake of sources of flavonoids.

We thank Walter Willett for use of his food-frequency questionnaire in this study; Aaron Folsom, Principal Investigator of the Iowa Women’s Health Study, for access to the data, and Bonniel Sceurman for technical assistance.

CGS and LMB mapped the foods in the food-frequency questionnaire to the foods in the flavonoid databases and conducted the statistical analyses; C-PH processed the original data and prepared the Iowa Women’s Health Study dataset that was used for the analysis; DRI, PIM, C-PH, LMB, and LH contributed to the design of the study and data analysis; LMB and PIM interpreted the results of the data analysis; PIM and CGS wrote the draft of the manuscript; and LH, C-PH, LMB, JAN, PJM, DRJ, and CGS contributed to the revision of the manuscript. None of the authors had any personal or financial conflict of interest.

REFERENCES

27. Feskanich D, Rimm EB, Giovannucci EL, et al. Reproducibility and


Dietary patterns, insulin resistance, and prevalence of the metabolic syndrome in women1–3

Ahmad Esmaillzadeh, Masoud Kimiagar, Yadollah Mehrabi, Leila Azadbakht, Frank B Hu, and Walter C Willett

ABSTRACT

Background: Although individual foods and nutrients have been associated with the metabolic syndrome, whether dietary patterns identified by factor analysis are also associated with this syndrome is not known.

Objective: We aimed to evaluate the association of major dietary patterns characterized by factor analysis with insulin resistance and the metabolic syndrome among women.

Design: Usual dietary intakes were assessed in a cross-sectional study of 486 Tehrani female teachers aged 40–60 y. Anthropometric and blood pressure measurements were performed, and fasting blood samples were taken for biomarker assessment. The metabolic syndrome was defined according to Adult Treatment Panel III guidelines, and insulin resistance was defined as the highest quartile of the homeostasis model assessment scores.

Results: We identified 3 major dietary patterns by factor analysis: the healthy dietary pattern, the Western dietary pattern, and the traditional dietary pattern. After control for potential confounders, subjects in the highest quintile of healthy dietary pattern scores had a lower odds ratio for the metabolic syndrome (odds ratio: 0.61; 95% CI: 0.30, 0.79; P for trend < 0.01) and insulin resistance (0.51; 0.24, 0.88; P for trend < 0.01) than did those in the lowest quintile. Compared with those in the lowest quintile, women in the highest quintile of Western dietary pattern scores had greater odds for the metabolic syndrome (1.68; 1.10, 1.95; P for trend < 0.01) and insulin resistance (1.26; 1.00, 1.78; P for trend < 0.01). Higher consumption of traditional dietary pattern was significantly associated only with abnormal glucose homeostasis (1.19; 1.04, 1.59; P < 0.05).

Conclusion: Significant associations exist between dietary patterns identified by factor analysis, the metabolic syndrome, and insulin resistance. Am J Clin Nutr 2007;85:910–8.

KEY WORDS Dietary patterns, metabolic syndrome, insulin resistance, factor analysis, women

INTRODUCTION

The term metabolic syndrome describes a clustering of risk factors for cardiovascular disease (CVD). Its pathophysiology is believed to include insulin resistance (1); but its definition is controversial (2). It is now established that this syndrome predicts the development of type 2 diabetes and CVD (3). Persons with the metabolic syndrome are also at greater risk of premature death due to CVD or all-cause mortality (4, 5). Cross-sectional and longitudinal epidemiologic studies have provided prevalence and incidence data on the syndrome, but estimates vary according to the criteria used (6, 7). The syndrome is common in the United States, particularly among Mexican Americans (8). However, it is not just that an epidemic of the metabolic syndrome is occurring in developed countries; rates of the metabolic syndrome in developing countries are also high. In Tehran, Iran, one-third of adults (9, 10) and one-tenth of the adolescent population (11) are affected.

The metabolic syndrome is a multifactorial disorder, and diet plays an important role in its development (12). Diet can be considered in terms of dietary patterns, an approach that has been used to investigate diet-disease relations (13–15). Dietary patterns address the effect of the diet as a whole and thus may provide insight beyond the effects described for single nutrients or foods (13).

Although several dietary factors have been associated with the metabolic syndrome (16–18), few studies have examined the association between dietary patterns and the metabolic syndrome. We are aware of only 2 reports that evaluated dietary patterns directly in relation to the metabolic syndrome (19, 20). Both used cluster analysis to identify dietary patterns, and it remains unknown whether dietary patterns identified by factor analysis are also associated with the metabolic syndrome. Factor analysis and cluster analysis are statistically different procedures, and each identifies dietary patterns with different food compositions (21). Although dietary patterns derived from both factor and cluster analysis have been associated with risk of chronic diseases (19–23), some evidence supports the possibility that a person’s dietary patterns would be best represented by using factor analysis (21, 24). The current study was conducted to assess the relation of major dietary patterns identified by factor...
analysis to insulin resistance and the metabolic syndrome in a group of Tehrani female teachers aged 40–60 y.

SUBJECTS AND METHODS

Participants

This cross-sectional study was conducted in a representative sample of Tehrani female teachers aged 40–60 y selected by a multistage cluster random-sampling method. The sample of 583 female teachers was invited to participate in the current study; 521 of the women agreed to do so. Participants with a history of CVD, diabetes, cancer, or stroke were excluded because of possible disease-related changes in diet. We also excluded women who had left >70 items blank on the food-frequency questionnaire (FFQ), who reported a total daily energy intake (EI) outside the range of 800–4200 kcal, and who were taking medications that would affect serum lipoprotein concentrations, blood pressure, and carbohydrate metabolism. These exclusions left 486 women for the current analysis.

Written informed consent was obtained from each participant. The study was approved by the research council of the National Nutrition and Food Technology Research Institute, Shahed Beheshti University of Medical Sciences.

Assessment of dietary intake

Usual dietary intake was assessed by using a 168-item semi-quantitative FFQ. All of the questionnaires were administered by a trained dietitian. The FFQ consisted of a list of foods with standard serving sizes commonly consumed by Iranians. 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. The reported frequency for each food item was then converted to a daily intake. Portion sizes of consumed foods were converted to grams by using household measures (25). Total EI was calculated by summing up EIs from all foods. Because of the large number of the food items relative to the number of participants, we assigned each food item into 1 of 41 defined food groups (Table 1). The basis for placing a food item in a certain food group was the similarity of nutrients. Some food items were considered individually as a food group because their nutrient profiles were unique (eg, eggs, margarine, coffee, and tea) or their consumption was considered to reflect a distinct dietary pattern [eg, garlic, broth, or doogh (an Iranian yogurt preparation with a consistency similar to that of whole milk)]. A previous validation study of this FFQ revealed good correlations between dietary intakes assessed by a similar FFQ and those from multiple days of 24-h dietary recalls completed during an earlier year-long study (26).

Assessment of anthropometric measures

Weight was measured while the subjects were minimally clothed and not wearing shoes; weight was measured with digital scales and recorded to the nearest 100 g. Height was measured by using a tape measure while the subjects were standing, were not wearing shoes, and had the shoulders in a normal position. Body mass index (BMI) was calculated as weight (in kg) divided by height (in m²). Waist circumference (WC) was measured at the narrowest level, and hip circumference was measured at the maximum level over light clothing, by using an unstretched tape

### TABLE 1

<table>
<thead>
<tr>
<th>Food groups</th>
<th>Food items</th>
</tr>
</thead>
<tbody>
<tr>
<td>Processed meats</td>
<td>Sausages</td>
</tr>
<tr>
<td>Red meats</td>
<td>Beef, hamburger, lamb</td>
</tr>
<tr>
<td>Organ meats</td>
<td>Beef liver</td>
</tr>
<tr>
<td>Fish</td>
<td>Canned tuna fish, other fish</td>
</tr>
<tr>
<td>Poultry</td>
<td>Chicken with or without skin</td>
</tr>
<tr>
<td>Eggs</td>
<td>Eggs</td>
</tr>
<tr>
<td>Butter</td>
<td>Butter</td>
</tr>
<tr>
<td>Margarine</td>
<td>Margarine</td>
</tr>
<tr>
<td>Low-fat dairy products</td>
<td>Skim or low-fat milk, low-fat yogurt</td>
</tr>
<tr>
<td>High-fat dairy products</td>
<td>High-fat milk, whole milk, chocolate milk, cream, high-fat yogurt, cream yogurt, cream cheese, other cheeses, ice cream</td>
</tr>
<tr>
<td>Tea</td>
<td>Tea</td>
</tr>
<tr>
<td>Coffee</td>
<td>Coffee</td>
</tr>
<tr>
<td>Fruit</td>
<td>Pears, apricots, cherries, apples, raisins or grapes, bananas, cantaloupe, watermelon, oranges, grapefruit, kiwi, strawberries, peaches, nectarine, tangerine, mulberry, plums, persimmons, pomegranates, lemons, pineapples, fresh figs and dates</td>
</tr>
<tr>
<td>Cruciferous vegetables</td>
<td>Cabbage, cauliflower, Brussels sprouts, kale</td>
</tr>
<tr>
<td>Yellow vegetables</td>
<td>Carrots</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>Tomatoes, tomato sauce, tomato pasta</td>
</tr>
<tr>
<td>Green leafy vegetables</td>
<td>Spinach, lettuce</td>
</tr>
<tr>
<td>Other vegetables</td>
<td>Cucumber, mixed vegetables, eggplant, celery, green peas, green beans, green pepper, turnip, corn, squash, mushrooms, onions</td>
</tr>
<tr>
<td>Legumes</td>
<td>Beans, peas, lima beans, broad beans, lentils, soy</td>
</tr>
<tr>
<td>Garlic</td>
<td>Garlic</td>
</tr>
<tr>
<td>Potatoes</td>
<td>Potatoes</td>
</tr>
<tr>
<td>French fries</td>
<td>French fries</td>
</tr>
<tr>
<td>Whole grains</td>
<td>Dark breads (Iranian), barley bread, popcorn, cornflakes, wheat germ, bulgur</td>
</tr>
<tr>
<td>Refined grains</td>
<td>White breads (lavash, baguettes), noodles, pasta, rice, toasted bread, milled barley, sweet bread, white flour, starch, biscuits</td>
</tr>
<tr>
<td>Pizza</td>
<td>Pizza</td>
</tr>
<tr>
<td>Snacks</td>
<td>Potato chips, corn puffs, crackers, popcorn</td>
</tr>
<tr>
<td>Nuts</td>
<td>Peanuts, almonds, pistachios, hazelnuts, roasted seeds, walnuts</td>
</tr>
<tr>
<td>Mayonnaise</td>
<td>Mayonnaise</td>
</tr>
<tr>
<td>Dried fruit</td>
<td>Dried figs, dried dates, dried mulberries, other dried fruit</td>
</tr>
<tr>
<td>Olive</td>
<td>Olives, olive oils</td>
</tr>
<tr>
<td>Sweets and desserts</td>
<td>Chocolates, cookies, cakes, confections</td>
</tr>
<tr>
<td>Hydrogenated fats</td>
<td>Hydrogenated fats, animal fats</td>
</tr>
<tr>
<td>Vegetable oils</td>
<td>Vegetable oils (except for olive oil)</td>
</tr>
<tr>
<td>Sugars</td>
<td>Sugars, candies, gaz (an Iranian confectionery made of sugar, nuts, and tamarisk)</td>
</tr>
<tr>
<td>Condiments</td>
<td>Jam, jelly, honey</td>
</tr>
<tr>
<td>Soft drinks</td>
<td>Soft drinks</td>
</tr>
<tr>
<td>Yogurt drink</td>
<td>Doogh</td>
</tr>
<tr>
<td>Broth</td>
<td>Broth</td>
</tr>
<tr>
<td>Salt</td>
<td>Salt</td>
</tr>
<tr>
<td>Pickles</td>
<td>Pickles</td>
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</tbody>
</table>
measure, without any pressure to body surface; measurements were recorded to the nearest 0.1 cm. Because the measurements were taken over light clothing, participants were asked to remove belts and tight or loose garments intended to alter the shape of the body, and the person measuring was asked to inspect the tension of the tape on the subject’s body to ensure that the tape had the proper tension—ie, neither too loose nor too tight. Although the narrowest waist is easy to identify in most persons, in some, no single narrowest waist can be identified because of a large amount of abdominal fat or extreme thinness (27). In the current study, when the narrowest point of the waist was difficult to identify (particularly in obese participants), we measured WC immediately below the end of the lowest rib, because in most persons the narrowest waist is at the lowest rib (27). To reduce error, all measurements were taken by the same technician.

Assessment of biomarkers

A blood sample was drawn between 0700 and 0900 into evacuated tubes after an overnight (12 h) fast. Blood samples were taken while the subject was sitting and according to a standard protocol; the samples were centrifuged for 10 min at 500 × g and at 4 °C within 30–45 min of collection. Samples were analyzed by using an autoanalyzer (Selectra 2; Vital Scientific, Spankeren, Netherlands). Fasting plasma glucose (FPG) was measured on the day of blood collection by the enzymatic colorimetric method and using glucose oxidase. Serum triacylglycerol concentrations were assayed with triacylglycerol kits (Pars Azmoon Inc, Tehran, Iran) by using enzymatic colorimetric tests with glycerol phosphate oxidase. HDL cholesterol was measured after precipitation of the apolipoprotein B–containing lipoproteins with phosphotungstic acid. Serum insulin concentrations were measured by using enzyme-linked immunosorbent assay kits and an enzyme-linked immunosorbent assay reader (Tecan Sunrise, Austria). The interassay and intraassay CVs of this method were <10%.

Assessment of blood pressure

For blood pressure measurements, participants were first asked to rest for 15 min. Then, a trained physician measured the blood pressure 3 times in seated participants by using a standard mercury sphygmomanometer, and thereafter the mean of 3 measurements was considered as the participant’s blood pressure. Systolic blood pressure was defined as the appearance of the first sound (Korotkoff phase 1), and diastolic blood pressure was defined as the disappearance of the sound (Korotkoff phase 5) during deflation of the cuff at a 2–3-mm/s rate of decrement of the mercury column.

Assessment of other variables

Data on physical activity were obtained by using an interview-based questionnaire and expressed as metabolic equivalent hours per week (MET-h/wk) (28). Additional covariate information regarding age, smoking habits, menopausal status, medical history, and current use of medications was obtained with questionnaires.

Definition of terms

The metabolic syndrome was defined as the presence of ≥3 of the following components as recommended by Adult Treatment Panel III (ATP III; 29): abdominal adiposity (WC >88 cm); low serum HDL cholesterol (<50 mg/dL); high serum triacylglycerol concentrations (≥150 mg/dL); elevated blood pressure (≥130/85 mm Hg); and abnormal glucose homeostasis (fasting plasma glucose ≥110 mg/dL). Insulin resistance was estimated on the basis of fasting glucose and insulin concentrations by using the homeostasis model assessment for insulin resistance (HOMA-IR) method (30) and was defined as the highest quartile of the HOMA-IR scores.

Statistical analysis

To identify major dietary patterns based on the 41 food groups, we used principal component analysis, and the factors were rotated by orthogonal transformation. The natural interpretation of the factors in conjunction with eigenvalues >1 and the Scree test (31) determined whether a factor should be retained. The Scree plot is a plot of the eigenvalues of derived factors. The eigenvalues of the factors dropped substantially after the third factor and remained more similar to each other after the fourth factor. The derived factors (dietary patterns) were labeled on the basis of our interpretation of the data and of the earlier literature. The factor score for each pattern was calculated by summing intakes of food groups weighted by their factor loadings (31), and each participant received a factor score for each identified pattern.

We categorized participants by quintile of dietary pattern scores. One-way analysis of variance with Tukey’s post hoc comparisons was performed to evaluate significant differences in general characteristics (eg, age, anthropometry, and physical activity) across quintile categories of dietary pattern scores; the distribution of qualitative variables across quintiles was evaluated by using chi-square tests. Age- and energy-adjusted means for dietary variables across quintiles of dietary pattern scores were calculated. We also calculated multivariate-adjusted means (ie, age, physical activity, smoking, menopausal status, total EI, and current estrogen use) for insulin and features of the metabolic syndrome. Analysis of covariance with Bonferroni correction was used to compare these means.

To determine the associations of dietary patterns with insulin resistance and the metabolic syndrome, we used multivariable logistic regression. First we obtained age-adjusted ORs, and then we adjusted for cigarette smoking (yes or no), physical activity (MET-h/wk), current estrogen use (yes or no), menopausal status (yes or no), and family history of diabetes and stroke (yes or no). We also adjusted for EI (kcal/d) in the third model, and finally we added BMI (kg/m²) to the logistic regression model to examine whether the relation was mediated by obesity. In all multivariate models, the first quintile of dietary patterns score was considered as a reference. To derive an estimate of association that better represents the relative risk, all ORs derived from logistic regression models were corrected by using the formula suggested by Zhang and Yu (32). The Mantel-Haenszel extension chi-square test was used to assess the overall trend of ORs across increasing quintiles of dietary pattern scores.

Because using cutoffs for defining the metabolic abnormalities involves some loss of information, we also studied relations between dietary pattern scores and metabolic risks as continuous variables by using partial correlation coefficients. All analyses were adjusted for age, EI, cigarette smoking, physical activity, current estrogen use, menopausal status, and family history of diabetes and stroke. In addition, we adjusted all models for BMI. We used SPSS software (version 9.05; SPSS Inc, Chicago IL) for all statistical analyses.
RESULTS

We identified 3 major dietary patterns by using factor analysis: the healthy dietary pattern (high in fruits, tomatoes, poultry, legumes, cruciferous and green leafy vegetables, other vegetables, tea, fruit juices, and whole grains), the Western dietary pattern (high in refined grains, red meat, butter, processed meat, high-fat dairy products, sweets and desserts, pizza, potatoes, eggs, hydrogenated fats, and soft drinks and low in other vegetables and low-fat dairy products), and the traditional dietary pattern (high in refined grains, potatoes, tea, whole-grains, hydrogenated fats, legumes, and broth). The factor-loading matrices for these dietary patterns are shown in Table 2. Other minor dietary patterns have also been identified by the factor analysis, but because of the small variances they explained, we did not consider them in the subsequent analyses.

Characteristics of the study participants across quintile categories of the dietary pattern scores are shown in Table 3. Compared with participants in the lowest quintile, those in the highest quintile of the healthy dietary pattern had significantly lower BMI and significantly lower prevalence of the metabolic syndrome, were significantly more physically active, and were significantly less likely to be obese. Conversely, in comparison with participants in the lowest quintile, those in the highest quintile of the Western dietary pattern had significantly higher BMI, were significantly less likely to exercise, and had significantly higher prevalence of obesity and the metabolic syndrome. Participants in the highest quintile of the traditional dietary pattern were significantly older, slightly more physically active, and significantly less likely to be obese than those in the lowest quintile. No significant difference was found in the distribution of current smokers and estrogen users across quintile categories of dietary patterns. Those in the highest quintile of the healthy dietary pattern had significantly lower intakes of energy and cholesterol and significantly higher intakes of vitamin B-6, magnesium, and fiber, whereas those in the highest quintile of the Western dietary pattern had significantly higher intakes of energy and cholesterol and significantly lower intakes of vitamin B-6, magnesium, and fiber. Participants in the highest quintile of the traditional dietary pattern had slightly lower EI than those in the lowest category, but their nutrient intakes were not significantly different in most cases.

ORs for the metabolic syndrome and insulin resistance across quintile categories of dietary pattern scores are presented in Table 4. After control for age, participants in the highest quintile of the healthy dietary pattern score had lower odds of the metabolic syndrome (OR: 0.55; 95% CI: 0.27, 0.74) and insulin resistance (0.47; 0.18, 0.89) than did those in the lowest quintile, whereas those in the highest quintile of the Western dietary pattern score had greater odds of the metabolic syndrome (OR: 2.67; 1.80, 3.96) and insulin resistance (0.79; 0.56, 1.13) than those in the lowest quintile. Further adjustment for other potentially confounding variables attenuated these associations, but they remained significant. Even after additional control for BMI, the inverse association of the healthy dietary pattern score and the positive association of that Western dietary pattern score with the metabolic syndrome remained significant. However, after adjustment for BMI, the positive association of the Western dietary pattern with insulin resistance disappeared. No significant overall associations were seen between the traditional dietary pattern score and the metabolic syndrome or insulin resistance.

In the multivariate models, participants in the highest quintile of the healthy dietary pattern score had lower odds for components of the metabolic syndrome (in the range of 0.50 for elevated blood pressure to 0.83 for abnormal glucose homeostasis) (Table 5). In contrast, those in the highest quintile of the Western dietary pattern score had significantly higher odds for components of the metabolic syndrome (in the range of 1.28 for low HDL cholesterol to 2.17 for elevated blood pressure). However, the association was not significant for abnormal glucose homeostasis (OR: 1.11; 0.95, 1.46). Although the trends of ORs across quintiles of the traditional dietary pattern score were significant in

### Table 2

Factor-loading matrix for major dietary patterns

<table>
<thead>
<tr>
<th>Food groups</th>
<th>Healthy</th>
<th>Western</th>
<th>Traditional</th>
</tr>
</thead>
<tbody>
<tr>
<td>Processed meats</td>
<td>—</td>
<td>0.39</td>
<td>—</td>
</tr>
<tr>
<td>Red meats</td>
<td>—</td>
<td>0.56</td>
<td>—</td>
</tr>
<tr>
<td>Organ meats</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Fish</td>
<td>0.22</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Poultry</td>
<td>0.53</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Eggs</td>
<td>—</td>
<td>0.35</td>
<td>—</td>
</tr>
<tr>
<td>Butter</td>
<td>−0.31</td>
<td>0.43</td>
<td>—</td>
</tr>
<tr>
<td>Margarine</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Low-fat dairy products</td>
<td>0.26</td>
<td>−0.37</td>
<td>—</td>
</tr>
<tr>
<td>High-fat dairy products</td>
<td>−0.23</td>
<td>0.39</td>
<td>—</td>
</tr>
<tr>
<td>Tea</td>
<td>0.39</td>
<td>—</td>
<td>0.42</td>
</tr>
<tr>
<td>Coffee</td>
<td>—</td>
<td>0.23</td>
<td>—</td>
</tr>
<tr>
<td>Fruit</td>
<td>0.74</td>
<td>−0.29</td>
<td>—</td>
</tr>
<tr>
<td>Fruit juices</td>
<td>0.37</td>
<td>0.21</td>
<td>—</td>
</tr>
<tr>
<td>Cruciferous vegetables</td>
<td>0.47</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Yellow vegetables</td>
<td>0.21</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>0.63</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Green leafy vegetables</td>
<td>0.41</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Other vegetables</td>
<td>0.71</td>
<td>−0.31</td>
<td>—</td>
</tr>
<tr>
<td>Legumes</td>
<td>0.52</td>
<td>—</td>
<td>0.26</td>
</tr>
<tr>
<td>Garlic</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Potatoes</td>
<td>0.29</td>
<td>0.35</td>
<td>0.46</td>
</tr>
<tr>
<td>French fries</td>
<td>—</td>
<td>0.24</td>
<td>—</td>
</tr>
<tr>
<td>Whole grains</td>
<td>0.34</td>
<td>—</td>
<td>0.40</td>
</tr>
<tr>
<td>Refined grains</td>
<td>—</td>
<td>0.66</td>
<td>0.51</td>
</tr>
<tr>
<td>Pizza</td>
<td>—</td>
<td>0.36</td>
<td>—</td>
</tr>
<tr>
<td>Snacks</td>
<td>—</td>
<td>0.29</td>
<td>—</td>
</tr>
<tr>
<td>Nuts</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mayonnaise</td>
<td>—</td>
<td>0.22</td>
<td>—</td>
</tr>
<tr>
<td>Dried fruit</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Olives</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Sweets and desserts</td>
<td>—</td>
<td>0.37</td>
<td>—</td>
</tr>
<tr>
<td>Hydrogenated fats</td>
<td>−0.20</td>
<td>0.34</td>
<td>0.28</td>
</tr>
<tr>
<td>Vegetable oils</td>
<td>—</td>
<td>0.20</td>
<td>—</td>
</tr>
<tr>
<td>Sugars</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Condiments</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Soft drinks</td>
<td>—</td>
<td>0.33</td>
<td>—</td>
</tr>
<tr>
<td>Yogurt drink</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Broth</td>
<td>—</td>
<td>—</td>
<td>0.23</td>
</tr>
<tr>
<td>Salt</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pickles</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Percentage of variance explained (%)</td>
<td>0.103</td>
<td>0.086</td>
<td>0.052</td>
</tr>
</tbody>
</table>

1 Values < 0.20 were excluded for simplicity.
### TABLE 3
Characteristics and dietary intakes of study participants by quintile (Q) categories of dietary pattern scores

<table>
<thead>
<tr>
<th></th>
<th>Healthy pattern score</th>
<th>Western pattern score</th>
<th>Traditional pattern score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q1 (lowest)</td>
<td>Q3 (highest)</td>
<td>Q5 (highest)</td>
</tr>
<tr>
<td></td>
<td>(n = 97)</td>
<td>(n = 97)</td>
<td>(n = 97)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>49 ± 6†</td>
<td>50 ± 7</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30.4 ± 3.4</td>
<td>27.8 ± 3.9</td>
<td>25.7 ± 3.8</td>
</tr>
<tr>
<td>WHR</td>
<td>0.91 ± 0.08</td>
<td>0.89 ± 0.08</td>
<td>0.85 ± 0.05</td>
</tr>
<tr>
<td>Physical activity (MET·h/week)</td>
<td>10.3 ± 9.1</td>
<td>14.7 ± 11.2</td>
<td>17.3 ± 10.8</td>
</tr>
<tr>
<td>Family history of diabetes (%)</td>
<td>9</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Family history of stroke (%)</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Current daily smoker (%)</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Obese (%)</td>
<td>47 ± 20</td>
<td>35 ± 20</td>
<td>20</td>
</tr>
<tr>
<td>Current estrogen use (%)</td>
<td>24 ± 26</td>
<td>26 ± 26</td>
<td>26</td>
</tr>
<tr>
<td>Metabolic syndrome (%)</td>
<td>37 ± 20</td>
<td>27 ± 20</td>
<td>20</td>
</tr>
<tr>
<td>Dietary intakes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total energy (kcal/d)</td>
<td>2675 ± 23</td>
<td>2341 ± 24</td>
<td>2052 ± 21</td>
</tr>
<tr>
<td>Carbohydrate (% of total energy)</td>
<td>59 ± 1</td>
<td>58 ± 1</td>
<td>56 ± 1</td>
</tr>
<tr>
<td>Protein (% of total energy)</td>
<td>10 ± 0.4</td>
<td>13 ± 0.4</td>
<td>14 ± 0.3</td>
</tr>
<tr>
<td>Fat (% of total energy)</td>
<td>31 ± 0.7</td>
<td>29 ± 0.6</td>
<td>28 ± 0.7</td>
</tr>
<tr>
<td>Cholesterol (mg/d)</td>
<td>191 ± 10</td>
<td>179 ± 8</td>
<td>150 ± 9</td>
</tr>
<tr>
<td>Dietary fiber (g/d)</td>
<td>12 ± 2</td>
<td>15 ± 1</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>Vitamin B-6 (mg/d)</td>
<td>0.7 ± 0.09</td>
<td>0.7 ± 0.1</td>
<td>1.1 ± 0.06</td>
</tr>
<tr>
<td>Magnesium (mg/d)</td>
<td>124 ± 3</td>
<td>149 ± 2</td>
<td>171 ± 3</td>
</tr>
</tbody>
</table>

† WHR, waist-to-hip ratio; MET, metabolic equivalent.

<table>
<thead>
<tr>
<th></th>
<th>Healthy pattern score</th>
<th>Western pattern score</th>
<th>Traditional pattern score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q1 (lowest)</td>
<td>Q3 (highest)</td>
<td>Q5 (highest)</td>
</tr>
<tr>
<td></td>
<td>(n = 97)</td>
<td>(n = 97)</td>
<td>(n = 97)</td>
</tr>
<tr>
<td>Obesity = BMI ≥ 30 kg/m².</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANOVA for quantitative variables and chi-square test for qualitative variables.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>± SD (all such values).</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Defined as the presence of ≥ 3 of the following components: 1) abdominal adiposity (waist circumference ≥ 88 cm); 2) low serum HDL cholesterol (&lt;50 mg/dL); 3) high serum triacylglycerol (≥ 150 mg/dL); 4) elevated blood pressure (≥ 130/85 mm Hg); 5) abnormal glucose homeostasis (fasting plasma glucose ≥ 110 mg/dL).</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>± SEM (all such values); adjusted for age and energy intake.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 4
Multivariate adjusted odds ratios (95% CIs) for metabolic syndrome across quintile (Q) categories of dietary pattern scores

<table>
<thead>
<tr>
<th>Metabolic syndrome</th>
<th>Healthy pattern score</th>
<th>Western pattern score</th>
<th>Traditional pattern score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q1 (n = 97)</td>
<td>Q3 (n = 97)</td>
<td>Q5 (n = 97)</td>
</tr>
<tr>
<td>Metabolic syndrome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolic syndrome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model IV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin resistance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model IV</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Defined as the presence of ≥3 of the following components: 1) abdominal adiposity (waist circumference ≥88 cm); 2) low serum HDL cholesterol (<50 mg/dL); 3) high serum triacylglycerol (≥150 mg/dL); 4) elevated blood pressure (≥130/85 mm Hg); 5) abnormal glucose homeostasis (fasting plasma glucose ≥110 mg/dL).

2 Adjusted for age.

3 Further adjusted for cigarette smoking, physical activity, current estrogen use, menopausal status, and family history of diabetes and stroke.

4 Additionally adjusted for energy intake.

5 Additionally adjusted for BMI.

6 Estimated on the basis of fasting glucose and insulin concentrations by using the homeostasis model assessment (HOMA-IR) method and defined as the upper quartile of the HOMA-IR scores.

### TABLE 5
Multivariate adjusted odds ratios (95% CIs) for components of the metabolic syndrome across quintile (Q) categories of dietary pattern scores

<table>
<thead>
<tr>
<th>Metabolic syndrome</th>
<th>Healthy pattern score</th>
<th>Western pattern score</th>
<th>Traditional pattern score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q1 (n = 97)</td>
<td>Q3 (n = 97)</td>
<td>Q5 (n = 97)</td>
</tr>
<tr>
<td>Abdominal adiposity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High triacylglycerol concentrations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elevated blood pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abnormal glucose homeostasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low HDL cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

7 Components of the metabolic syndrome were defined as 1) abdominal adiposity (waist circumference ≥88 cm); 2) low serum HDL cholesterol (<50 mg/dL); 3) high serum triacylglycerol (≥150 mg/dL); 4) elevated blood pressure (≥130/85 mm Hg); and 5) abnormal glucose homeostasis (fasting plasma glucose ≥110 mg/dL). Adjusted for age, cigarette smoking, physical activity, current estrogen use, menopausal status, family history of diabetes and stroke, and energy intake.
most cases, the highest category of this dietary pattern was associated significantly only with abnormal glucose homeostasis (OR: 1.19; 1.04, 1.59).

After adjustment for potential confounding variables in partial correlation analysis, the healthy dietary pattern score was associated positively with serum HDL cholesterol and inversely with other metabolic variables (Table 6). Even when the models were further adjusted for BMI, all associations remained significant except the positive association of this score with HDL cholesterol and its inverse association with plasma glucose. The Western dietary pattern score also was independently related to various metabolic risk factors except plasma glucose concentration. These associations were inverse for HDL cholesterol and positive for other metabolic variables. When we further adjusted for BMI, the associations of this pattern score with WC and HDL cholesterol disappeared. The traditional dietary pattern score was not significantly associated with any metabolic variables (Table 6).

### DISCUSSION

As stated in Results, we observed 3 major dietary patterns in this population: the healthy dietary pattern, the Western dietary pattern, and the traditional dietary pattern. Further analysis suggested that these major dietary patterns are related to insulin resistance and features of the metabolic syndrome. The healthy dietary pattern was associated with lower risks of insulin resistance and the metabolic syndrome, whereas the Western dietary pattern was associated with higher risks of insulin resistance and the metabolic syndrome. We found no significant association between the traditional dietary pattern and these conditions. All associations were independent of other lifestyle factors. To our knowledge, this is the first investigation in which major dietary patterns identified by factor analysis have been associated directly with the metabolic syndrome.

Although insulin resistance and the metabolic syndrome are underlying causes of major chronic diseases (33), few studies have assessed dietary patterns in relation to these conditions. In the Framingham Offspring Study (20), higher and lower prevalences of the metabolic syndrome have been reported in women with the “empty calorie” and the “wine and moderate eating” dietary patterns, respectively. In the Malmö Diet and Cancer Cohort (19), features of the metabolic syndrome were more prevalent in women with the “white-bread” dietary pattern and less prevalent in women with the “milk-fat” pattern. In a cross-sectional study in a British population (34), a dietary pattern characterized by high consumption of fruit and vegetables and low consumption of processed meat and fried foods was inversely associated with features of the metabolic syndrome. However, that study was limited by lack of control for physical activity, which tends to be associated with dietary patterns (35). Data on the association of dietary patterns with insulin resistance or insulin sensitivity are sparse, as they are for an association with the metabolic syndrome. Whereas some studies have considered the association with insulin sensitivity as their main objective (36), others have reported it as an accessory finding (37, 38).

In a cross-sectional study of a multiethnic cohort of 980 subjects aged 40–69 y, Liese et al (36) found that subjects with the “white bread” pattern (identified by cluster analysis and high in whole grains, vegetables, potatoes, low-fat milk, fish, nuts, and tofu) had lower levels of insulin sensitivity, whereas those with the “dark bread” pattern (with high intakes of dark bread and high-fiber cereal, rice and pasta, cruciferous vegetables, other vegetables, potatoes, low-fat milk, fish, nuts, seeds, and tofu) and with the “wine” pattern (with high intakes of wine and mixed drinks) had greater insulin sensitivity than did subjects with the other patterns identified. Higher scores on the Western dietary pattern (identified by factor analysis) were associated with higher insulin concentrations in the Health Professionals Follow-up Study (37). The same results have also been observed in the third National Health and Nutrition Examination Survey (38) and in an Irish population (39).

As was found in other studies, we found in the current study that the healthy dietary pattern is associated with lower risk of...
metabolic abnormalities, whereas the Western dietary pattern is related to higher risk of adverse metabolic risk factors. The inverse association between the healthy dietary pattern and the metabolic syndrome could be attributed to that pattern’s healthy constituents, including whole grains (17), fiber (40), fruit and vegetables (40, 41), and magnesium (42). The mechanisms by which greater intakes of these foods and nutrients may contribute to the inverse association between the healthy dietary pattern and the metabolic syndrome are not fully understood, but they are likely to be many (17, 40–42). Constituents of fruit, vegetables, and whole grains, including dietary fiber, vitamin E, folate, and magnesium, have been independently associated with reduced metabolic risks related to metabolic syndrome. An additional protective effect of other constituents of these foods or their interactions may also explain their beneficial effects. Reduced insulin demand may be another protective mechanism associated with higher intakes of these foods. In general, because of their physical form and their viscous fiber content, these foods tend to be slowly digested and absorbed, and thus they have relatively low glycemic indexes. Furthermore, most foods in the healthy dietary pattern have a low glycemic load, which has been documented to be associated with lower risk of insulin resistance (43).

The healthy dietary pattern we identified in the current study is somewhat similar to the patterns that have been labeled “prudent dietary pattern” in other studies (44, 45). Our healthy dietary pattern was also similar to the Dietary Approaches to Stop Hypertension eating plan, which has been recommended for decreasing blood pressure (46) and improving features of the metabolic syndrome (47). The positive association between the Western dietary pattern and the metabolic syndrome could be attributed to the lower amounts of beneficial foods and nutrients that this pattern contains. Higher intakes of refined grains (17) and saturated fat (48) in this dietary pattern also could explain part of this association. We observed no association between the traditional dietary pattern and the risk of the metabolic syndrome or insulin resistance. The complex nature of this pattern may explain this finding to some extent. This dietary pattern was loaded with both healthy (whole grains, tea, and legumes) and unhealthy (refined grains, potatoes, and hydrogenated fats) foods. Whereas healthy foods of this pattern have been reported to be protectively associated with the metabolic syndrome (17, 49, 50), the pattern’s unhealthy constituents have adverse effects on metabolic markers (17, 48, 51).

Some of the relations remained even after control for BMI. This shows that general obesity cannot explain all associations between diet and chronic diseases and that other factors, such as abdominal adiposity, may be responsible. We have not controlled for WC, as a measure of abdominal adiposity, in our analysis, because abdominal adiposity is one feature of the metabolic syndrome. However, our previous investigation in Tehran women showed that WC is a better index than BMI to be used in explaining metabolic abnormalities (52).

The dietary pattern approach is complementary to analyses using individual foods or nutrients, which are limited by biologic interactions and colinearity among nutrients. The logic behind the dietary pattern approach is that foods and nutrients are not eaten separately but are eaten in the form of specified dietary patterns. However, all statistical methods that have been used for data reduction have limitations. For example, using factor analysis for dietary data reduction has been criticized for its subjectivity in nature and for the difficulty of replicating the results in other populations (53). However, similar dietary patterns derived by factor analysis have been observed in different populations. It appears that the dietary patterns observed in this Iranian population are similar to those in Western populations. This is not surprising, because, during the past few years, Iran has experienced a socioeconomic transition coupled with westernization in diet and lifestyle (54, 55).

Several limitations need to be considered in the interpretation of our findings. We assessed dietary patterns by using food intake data only, whereas the inclusion of eating behaviors such as meal and snack patterns in dietary pattern analysis has been recommended (56). Limitations of the FFQ also apply to dietary pattern analyses that are based on dietary information collected by this method. The other limitation of our study is its cross-sectional nature. Thus, the association between these dietary patterns and the metabolic syndrome remains to be confirmed in prospective analyses. We cannot generalize our findings to all Iranian populations, because, in Iran, teachers have a socioeconomic status higher than that of the general population. However, participants in the current study were selected from 4 large, socioeconomically diverse districts of Tehran, so that a broad range of dietary habits were represented.

In conclusion, the current findings indicate that a dietary pattern characterized by high consumption of fruit, vegetables, poultry, and legumes is associated with reduced risk of insulin resistance and the metabolic syndrome in Tehran female teachers. In contrast, a dietary pattern with high amounts of refined grains, red meat, butter, processed meat, and high-fat dairy products and low amounts of vegetables and low-fat dairy products is associated with a greater risk of the metabolic syndrome.

We thank the participants of the study for their enthusiastic support. None of the authors had any personal or financial conflicts of interest.

AE and LA designed the study, collected and analyzed the data, and wrote the manuscript; MK served as a supervisor and YM as advisor for this research; and FBH and WCW reviewed the study and contributed to manuscript preparation.

REFERENCES


n−3 Fatty acids and the endocannabinoid system

Dear Sir:

Carpentier et al (1) mentioned in a recent issue of the Journal that the pleiotrophic effects of n−3 polyunsaturated fatty acids (PUFAs) decrease the burden of the metabolic syndrome, which prevails in Western countries and is related to the epidemic of obesity. In large epidemiologic studies, persons who consume higher amounts of fish also consume higher amounts of total calories but are not more obese, although the levels of exercise are similar in both Western countries (2, 3) and in Japan (4). Previously, I speculated that, at least in part, the beneficial effects of fish oil on obesity and obesity-related metabolic risk factors may be related to long-chain monounsaturated fatty acids (5), which may have relatively high ligand activities on peroxisome proliferator–activated receptor delta (6). Recently, Horvath (7) reviewed the effects of the endocannabinoid system on energy homeostasis and pointed out that the initial anorectic effect of the cannabinoid receptor 1 (CB1) antagonist, rimonabant, is diminished after the first weeks, whereas longer lasting weight loss is achieved. These findings indicate that the peripheral metabolic actions of cannabinoids are very important in body weight regulation. It is possible that n−3 PUFAs may act as competitive inhibitors in the peripheral endocannabinoid system, thereby promoting energy metabolism and exerting antiobesity and antiinflammatory effects. Two major CB1 agonists—anandamide (arachidonoyl ethanolamine) and 2-arachidonoylglycerol—are n−6 PUFAs derivatives, whereas n−3 and n−6 PUFAs are competitors as components of cell membrane phospholipids and in many biochemical pathways, such as eicosanoids and leukotrienes. At least in the brain of mice, n−3 PUFAs deficiency elevates and n−3 PUFAs enrichment reduces 2-arachidonoyl glycerol concentrations (8).

The author had no conflict of interest.

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α-Linolenic acid and fish oil n–3 fatty acids and cardiovascular disease risk

Dear Sir:

In evaluating the cardiovascular merits of n–3 fatty acids, Wang et al (1) appropriately sought the best available evidence and used randomized controlled human trials (RCTs) as their benchmark. As they showed, such trials designed to examine the cardiovascular effects of α-linolenic acid (ALA) have not been done to everyone’s satisfaction. Because they clearly defined at the outset what they meant by “high-quality evidence,” we cannot disagree with their conclusion there is no “high-quality evidence to support a beneficial effect of ALA.” What we reject however is that, once they make this initial point, Wang et al slide down a slippery slope toward broad enthusiasm for fish oils and outright dismissal of ALA. Neither position is supported by the existing literature.

First, it is incorrect to say that Dolecek’s analysis of the MRFIT study (2) showed no association between ALA and cardiac death; Dolecek’s analysis showed that, as a percentage of energy intake and in g/d, ALA was significantly negatively associated with cardiovascular, cancer, and all-cause mortality. Second, acute, short-term experiments showed that ALA has antiarrhythmic effects (3) and reduces platelet aggregation (4), and both effects could plausibly contribute significantly toward reduction of cardiovascular and all-cause mortality. Third, for all its possible confounders, the Lyon study (5) was a randomized controlled secondary prevention trial that, supported by a blood fatty acid analysis, clearly implicated ALA in risk reduction of cardiovascular disease and death. Hence, these diverse examples are consistent with cardiovascular benefits of ALA. They can in no way substitute for placebo-controlled RCTs, but they show that grounds exist for well-controlled trials to assess whether ALA reduces the risk of cardiovascular death.

Furthermore, Wang et al cite the concern with regard to ALA and prostate cancer in the absence of confirmatory RCT evidence but downplay some potentially equally important adverse cardiovascular effects of fish oils where RCTs exist. For instance, they cite the recent study by Raitt et al (6), which was conducted in subjects with an implantable cardioverter defibrillator, and mention that the risk of death did not change but downplayed the significantly increased risk of ventricular tachycardia or ventricular fibrillation when consuming 1.3 g fish oil n–3 fatty acids/d. At the time of publication of the article by Wang et al, they may not have been aware that Frost and Vestergaard (7) showed in a population study that Danes who consumed 1.29 g fish oil n–3 fatty acids/d (the top quintile) had a 34% higher rate of atrial fibrillation than did those who consumed 0.16 g fish oil n–3 fatty acids/d (the bottom quintile).

We are not saying that these reports of adverse cardiovascular outcomes with consumption of fish oils constitute sufficient evidence to dismiss the beneficial effects seen in controlled trials. We are saying that a systematic review purporting to give an “evidence-based review” of the cardiovascular effects of n–3 fatty acids should not conflate an absence of well-controlled trials examining cardiovascular effects of ALA with an absence of evidence that ALA has any benefits for the cardiovascular system. Furthermore, not all would agree that the arrhythmogenic effects of fish oils in certain cardiac patients are “minor;” the adverse effects of all n–3 fatty acids should be given appropriate and similar scrutiny. Clearly, additional ALA trials are overdue considering the strength of the existing evidence and the seriousness of the disease.

None of the authors had any conflicts of interest.

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TABLE 1
Fatty acid pattern in tissue phospholipids of n–3 fatty acid–depleted and control female rats

<table>
<thead>
<tr>
<th></th>
<th>% by wt</th>
<th></th>
<th>% by wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control rats</td>
<td>n–3 Depleted rats</td>
<td>Control rats</td>
</tr>
<tr>
<td>20:4n–6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>12.04 ± 0.30 (16)</td>
<td>18.00 ± 0.30 (18)²</td>
<td>0.23 ± 0.03 (16)</td>
</tr>
<tr>
<td>Liver</td>
<td>29.52 ± 0.34 (16)</td>
<td>38.31 ± 0.26 (18)²</td>
<td>0.17 ± 0.02 (16)</td>
</tr>
<tr>
<td>Soleus muscle</td>
<td>18.67 ± 0.26 (16)</td>
<td>28.82 ± 0.25 (18)²</td>
<td>0.22 ± 0.07 (16)</td>
</tr>
<tr>
<td>Heart muscle</td>
<td>22.93 ± 0.31 (16)</td>
<td>32.33 ± 0.42 (18)²</td>
<td>0.17 ± 0.03 (16)</td>
</tr>
<tr>
<td>Endocardium</td>
<td>24.20 ± 0.38 (3)</td>
<td>31.54 ± 0.50 (11)²</td>
<td>0.40 ± 0.05 (3)</td>
</tr>
</tbody>
</table>

1 All values are ± SE; n in parentheses.
² Significantly different from control rats, P < 0.005.
Letters to the Editor

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References


Reply to E Vos et al

Dear Sir:

We thank Vos et al for their comments on our article (1), but we disagree with their statement that our review showed “broad enthusiasm for fish oil and outright dismissal of ALA [α-linolenic acid].” Our systematic review focused on the health effects of dietary n-3 fatty acids on clinical cardiovascular outcomes in humans and evaluated available evidence according to predefined questions. To minimize bias, conclusions were drawn based on the studies that met predefined criteria.

Vos et al did not disagree with the criteria we used to determine the quality of evidence. However, they seem to differ in what they would consider to be valid evidence by referencing several studies that did not meet our predetermined criteria. They refer to an acute, short-term experimental study in dogs to illustrate the potential beneficial antiarrhythmic effects of α-linolenic acid (ALA) (2). In the study by Billman et al (2), ALA was infused intravenously in an exercise-ischemia model of ventricular fibrillation. In fact, we reviewed this study in a separate article addressing the question of the effect of n-3 fatty acids on selected arrhythmia outcomes in animal models (3). Even if evidence from a dog study could be used to infer a benefit in humans, the experimental setting is highly unphysiological. Furthermore, whether such high plasma ALA concentrations are even achievable in a human consuming ALA is unknown (however, it is very unlikely).

Vos et al also cited the study by Freese (4), in which ALA supplementation reduced in vitro measures of platelet aggregation, as evidence that the reduction in platelet aggregation plausibly contributed to a reduction in the risk of cardiovascular events. In this study, very high intakes of both ALA (6 g/d) and eicosapentaenoic acid + docosahexaenoic acid (EPA + DHA; 5.2 g/d) were provided, neither of which has been shown at these doses to be cardioprotective. In addition, no effects were seen on plasma lipids for either ALA or EPA + DHA, and the minor effects on platelet aggregation do not explain the reduction in cardiac events that have been observed with ≤1 g EPA + DHA.

The Lyon Heart Study (5) was not designed to show and cannot be construed as showing that ALA was the agent responsible for the reduction in clinical events. Multiple variables were manipulated in that trial. Simply because serum concentrations of ALA were inversely associated with a reduced risk does not indicate that ALA that was responsible for the reduced risk. Association is not cause and effect, and a “true–true-and-unrelated” association is always possible.

Experimental studies conducted in animals and in vitro studies conducted in humans that focus on intermediate outcomes are important to uncover the mechanisms involved in the potential beneficial cardiovascular outcomes of n-3 fatty acid consumption in humans. The findings in the articles referred to by Vos et al support the hypothesis that ALA is cardioprotective and are reinforced by recent epidemiologic data (6, 7). However, these promising results must still be directly tested in human randomized controlled trials, rather than the benefits to humans assumed. Whether ALA has similar beneficial cardiovascular effects through diet or as supplements remains to be explored. Direct evidence does not support the view that ALA reduces the risk of cardiovascular events.

JL and AHL have no conflict of interest to declare. WSH is a scientific advisor to OmegaMetrix LLC, Monsanto, CardioTabs, and TherRx.

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References


New horizons for glycemic index research

Dear Sir:

A recent editorial by Pereira (1) in the Journal presented several important perspectives on studies of the glycemic index (GI) and glycemic load in relation to weight status. Most of these perspectives related to the interpretation of findings reported thus far from epidemiologic studies, and Pereira looks forward to studies yet to be conducted. It is important also to recognize that, in addition to various analytic issues, numerous potentially serious methodologic problems exist with respect to the application of the GI to studies of usual diet; these problems have to do with the index itself. Such concerns were noted both during the 1980s (2) and more recently (3). They include, but are not limited to, the derivation of the underlying GI values for specific foods from studies of glucose excursion in response to ingestion of the food after an overnight fast, whereas much of the food consumed as part of a usual diet is consumed during the postprandial interval. And, as Pereira appropriately noted, habitual diets that have a low GI may simply be generally prudent diets, with frequent consumption of nutrient-rich and fiber-rich foods, as recently described (4). Dietary fiber, the type of carbohydrate, and the processing of carbohydrate-containing food do seem to matter to glucose and insulin metabolism and related health outcomes. It is extremely important that our understanding of these processes is advanced. Pereira noted inconsistencies in the literature and called for longer, high-quality, randomized controlled trials. I suggest that, before such trials are conducted, new work be conducted to address methodologic problems and to advance our understanding of the underlying construct of the GI. Without further work to better characterize aspects of carbohydrate-containing foods that affect health, we risk spending limited research dollars to produce studies that will continue to be sometimes positive and sometimes negative, but we will still lack the underlying knowledge required to understand those findings.

The author had no conflict of interest.

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REFERENCES


Reply to EJ Mayer-Davis

Dear Sir:

Mayer-Davis feels that further methodologic research is needed to better understand the glycemic index (GI) before moving forward with more definitive randomized trials on GI and body weight regulation and related health outcomes. As evidence that GI has questionable clinical importance in free-living settings, she refers to her null findings on associations between GI derived from a food-frequency questionnaire (FFQ) and measures of fasting and postprandial glycemia in a relatively small study of a multiethnic cohort (1). However, the use of glycemic endpoints, as attractive as they are for characterizing diabetes risk, does nothing to overcome the shortcomings of the FFQ that I pointed out in my editorial (2) on the study by Hare-Bruun et al (3). Whether the endpoint is body weight, serum glucose concentration, glycosylated hemoglobin, or frank diabetes or any other malady, if dietary intake is assessed with an instrument of dubious validity, a good chance exists that the finding will be null and uninformative. Indeed, the validation study of the FFQ used in the study described by Mayer-Davis revealed correlation coefficients for total carbohydrate, between the FFQ and eight 24-h dietary recalls, ranging from 0.25 to 0.64 across ethnic groups. Overall, a validity correlation of 0.37 was noted for energy-adjusted, log-transformed carbohydrate. That is, the FFQ-derived carbohydrate intake explained only 14% of the variance in the 24-h recall-derived carbohydrate intake (4). Bias toward the null and negative publication bias are rampant in secondary data analysis of existing cohort studies. Certainly, future studies should aim to test the validity of GI and glycemic load (GL) from FFQ- and diet history–based data. My strong suspicion is that the validity correlation will be similar to that for total carbohydrate—somewhere on the order of 0.4 to 0.6.

Several fruitful, well-controlled intervention studies on the topic of GI and body weight regulation have been conducted in the recent past (5–9). Although not entirely consistent in their findings, these studies laid the groundwork and provided the preliminary evidence in support of larger and longer studies. Whereas more contributions may come from basic food chemistry studies that attempt to learn more about the effect of specific starches, sugars, and fibers on postprandial glycemia, it would not be prudent to lose the momentum we have gained from the more applied, clinical intervention studies. A recent randomized controlled feeding trial conducted by McMillan-Price et al (5) examined daily variations in blood glucose...
and insulin from morning to evening in 129 subjects randomly assigned to high- and low-GI diets at different carbohydrate and protein concentrations. GI had a direct and important effect on the 10-h blood glucose and insulin profiles, independent of amount of carbohydrate and protein in the diet. The study of McMillan-Price et al provided evidence in support of Mayer-Davis’s concern that the effect of GI on glycemia may be attenuated later in the day, likely as a result of the effect of previous meals and variations in physical activity. However, large and statistically significant differences in the 10-h area under the curve for glucose and insulin were observed, which documented the important aggregate effect of a GI that was based on several meals and snacks fed from morning to evening in a manner mimicking free-living conditions (5). Over the course of the intervention, the low-GI diet was observed to have beneficial effects on body fat changes and on some risk factors for cardiovascular disease (5). Similar effects on changes in disease risk factors were noted in a weight-loss intervention isocalorically comparing high- and low-GL diets (6). In addition, although the expected 10% decrease in resting energy expenditure (the main outcome of the study) was observed during 10% weight loss with the high-GL diet, that change was attenuated to 5.5% with the low-GL weight-loss treatment (P < 0.05 for treatment effect; 6). Collectively, these studies clearly show that GI and GL are valid concepts in mixed meals in real-world, free-living settings. Some larger and longer, hypothesis-driven, controlled intervention studies of the effect of GI and GL on body weight regulation and related chronic disease risks are ready and waiting to be conducted, and some likely are in progress.

The author had no personal or financial conflict of interest.

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REFERENCES
I felt a sense of déjà vu when I started to examine and read this latest publication from the National Academies Press on the Dietary Reference Intakes (DRIs). Those of us who remember previous versions have long yearned for the good old days when the dietary standards were in one volume and easily obtained by walking to our bookshelf. A protracted revision of the dietary standards has now given rise to the DRIs, which has been published over several years in several volumes. This book summarizes the basis of the new paradigm, presents the statistical foundations of such, and reviews, for each nutrient, how the Estimated Average Intake, the Recommended Dietary Intake, the Adequate Intake, and the Upper Level of intake were derived. Each nutrient is addressed in its own chapter, and all of the chapters have similar outlines and formats, which make them easy to read. There are 3 parts to the book: part 1 concerns the development and application of the DRIs; part 2 focuses on energy, macronutrients, water, and physical activity; and part 3 addresses vitamins and minerals.

Most of the chapters begin by explaining the function of the nutrient in the body (function, absorption, metabolism, storage, and transport) and are followed by a section on how the DRIs were determined, what special considerations were taken in their determination, food sources (including supplements) and bioavailability, and inadequate and excess intakes. Each chapter concludes with a checklist of “Key Points.”

A shortcoming of the book is that it does not contain references; however, the reader is referred to the volumes of earlier works in which citations are available and Web addresses are given. Given the new age of computers and technology, it appears that the days of flipping to the end of a chapter to locate references to the cited works are over. An additional weakness is the overemphasis of scientific results from the authors’ own laboratories to the exclusion of other important work in the field. There is also a lack of information that is useful in bringing together the disparate topics covered in the book. How the various dietary components interact is not discussed adequately, except in a brief chapter on how dietary components protect against cancer. This chapter does not fully explain any of the mechanisms that are targeted, such as apoptosis, proliferation, or checkpoint regulation. Instead, where full chapters are needed, only brief paragraphs over a few pages are provided. This overview is wholly inadequate for researchers and too brief and full of jargon for the uninitiated reader. Therefore, researchers, including fellows and faculty interested in the field of nutrition and cancer, would be better served through careful reading of available review articles on these topics. Those new to the field would be better off finding a more definitive and clearly written text on this subject.

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This modest volume is entitled Nutrition and Cancer Prevention; however, it has virtually no clinical information useful for uninitiated health care practitioners or researchers outside the field of nutrition and cancer. The book is a compilation of fairly brief chapters on various substances that are classified as dietary components, including vitamins, carotenoids, synthetic vitamin analogs, minerals (calcium and selenium), phytosterols, polyphenols, isothiocyanates, and lipids (n–3 fatty acids, conjugated linoleic acid, and sphingolipids), with brief chapters on obesity and alcohol as risk factors for cancer. The list is neither adequately complete nor adequately detailed in its parts. Although some of the chapters, such as the n–3 fatty acids and colon cancer chapters by BS Reddy and the flavonoid chapter by J-R Zhou, are adequate, many others are overly brief and difficult to read. They are dense with references and often do not adequately explain the findings being discussed in a manner comprehensible to the reader. An additional weakness is the overemphasis of scientific results from the authors’ own laboratories to the exclusion of other important work in the field. There is also a lack of information that is useful in bringing together the disparate topics covered in the book. How the various dietary components interact is not discussed adequately, except in a brief chapter on how dietary components protect against cancer. This chapter does not fully explain any of the mechanisms that are targeted, such as apoptosis, proliferation, or checkpoint regulation. Instead, where full chapters are needed, only brief paragraphs over a few pages are provided. This overview is wholly inadequate for researchers and too brief and full of jargon for the uninitiated reader. Therefore, researchers, including fellows and faculty interested in the field of nutrition and cancer, would be better served through careful reading of available review articles on these topics. Those new to the field would be better off finding a more definitive and clearly written text on this subject.

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The use of dietary supplements in the United States and the United Kingdom is widespread, and it is also a growing practice in other industrialized countries. Many books and websites provide detailed descriptions of the various ingredients in supplements and advice on use, serving primarily as reference works to be consulted for specific information as needed. Geoffrey P Webb, a Senior Lecturer in Nutrition and Physiology at the University of East London (London, United Kingdom), takes a different approach in this book. He has provided a brief introduction to supplements, which is meant to be read from cover to cover.

The book’s first chapter provides an overview of the growing use of supplements and the reasons for their use, the relative merits of various study methods to evaluate supplement efficacy and safety, the legal definitions of supplements, and the regulation of these products in the United States and United Kingdom. The second chapter includes a discussion of micronutrient adequacy, emphasizing the Reference Nutrient Intake (RNI) standard in the United Kingdom and secondarily the Recommended Dietary Allowance (RDA) of the United States [but without a discussion of the United States’ Dietary Reference Intakes (DRIs)].

Most of the book examines individual vitamins, minerals, fatty acids—what the author calls “nonessential ‘nutrients’”—ie, glucosamine, chondroitin, S-adenosyl-l-methionine, choline, lecithin, carnitine, creatine, coenzyme Q10, lipoic acid, and methylsulfonylmethane—and 17 “plant and animal extracts,” including ginkgo, chitosan, and echinacea. Each substance’s basic biochemistry, effects on human metabolism, recommended intakes, and provenance are explained, and one or more health-related claims—eg, ginseng for psychological well-being, athletic performance, and cancer prevention—are briefly evaluated.

The book’s best chapter provides a balanced discussion of free radicals, the oxidant theory of disease, mechanisms to limit free-radical damage, antioxidants in food, and the potential benefits and risks of antioxidant supplements.

This book’s title suggests that functional foods receive substantial attention, but such is not the case. The final chapter addresses these products in a cursory way, primarily as a vehicle to discuss sterols and stanols, phytoestrogens (primarily from soy), and probiotics and prebiotics—ingredients that are also available in supplements. Dietary Supplements and Functional Foods appears to be intended for health science students, at the college level and beyond, who, Webb says, “have limited specialist nutritional background.” However, it cannot serve as a well-rounded introductory text on the subject without providing extensive supplementary materials on such topics as the regulation of supplements and issues of product quality and labeling. The book suffers from the inclusion of dated information on nutrient intakes and supplement use, and the reference list both is relatively small and omits superior and more up-to-date citations. Readers may be disappointed in the book’s small size, given the broad and multifaceted nature of the topic, the high price, the emphasis on British nutrient standards and dietary or supplement patterns, and the degree of expertise of the author, who began to seriously study dietary supplement issues only ≈5 y ago.

The author had no personal or financial conflict of interest with the subject of this book or with the book’s author.

Paul R Thomas


Nutritional Strategies for the Diabetic & Prediabetic Patient, edited by Jeffrey I Mechanick and Elise M Brett, is one of several recently published books addressing the subject of diabetes and obesity. The proliferation of books that cover these subjects reflects the current interest by physicians and health care personnel in the pandemic of these diseases. Most nations in Europe, Asia, and South America report a rising prevalence of diabetes, with devastating consequences for the patient and a great burden placed on health care resources. The preface of this book states that it was written primarily to advance physicians’ knowledge of nutrition as it relates to diabetes and to enable them to provide evidence-based recommendations to their diabetic patients. However, the book falls short of accomplishing these goals.

The book comprises 368 pages; the 15 chapters and 6 appendices were written by 22 contributors, 9 of whom are from Mount Sinai School of Medicine (New York, NY). The chapters vary in quality and depth; some provide lucid descriptions of the subject, and some are rather superficial in nature. Most authors did not review the topics in the detail needed to provide a reference resource to physicians or they did not provide sufficient practical information to aid those in the daily practice of medicine in the care of their patients; in some cases, neither of those goals was met.

The chapter entitled “Mitochondrial function in diabetes: pathophysiology and nutritional therapeutics,” which has 389 references, provides an excellent in-depth review of the subject. The chapter entitled “Nutritional strategies for wound healing in diabetic patients,” which has 216 references, is unique and informative, whereas the chapter entitled “Carbohydrate counting” falls short. Indeed, it is very short: 5 pages and 7 references. The appendices of the book do not add value for the reader.

A great need exists for information about diabetes and for methods that will help prevent or treat diabetes (or both). Nutritional strategies are indeed needed. Approximately 20–21 million Americans—≈7% of the US population—have the disease.
In approximately one-third of diabetic persons, the disease may be underdiagnosed. After a decade of established diabetes, >20% of diabetes patients have had a cardiovascular event, 5% develop blindness, and 1–2% experience end-stage renal disease or lower-extremity amputation. Nearly 95% of diabetes patients have type 2 diabetes, which is closely linked to obesity.

This book lacks an integrative view and does not provide a coherent plan for a nutritional management. Nor does it set forth the strategies that would help deal with the prevention of diabetes or the interventions required to treat obesity and diabetes.

The author had no personal or financial conflict with the topic of this book or with its editors.

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The American Dietetic Association Complete Food and Nutrition Guide, a comprehensive, well-indexed handbook, includes a wide range of nutrition and food topics. It is aimed at the interested public and is written in a quick reference style and is sprinkled with self-assessment tools for each topic. These assessment tools pull the reader into the topic, make the information applicable to everyday life, and help identify areas in which more information is necessary. Of the 676 pages, 630 are textual, which are followed by list of resources, 28 pages of appendixes (mostly tables), and a complete index.

Topics are presented in an interesting order and not in the traditional manner. The first section discusses fitness and body weight, fats, vitamins and minerals, carbohydrates, fiber, and water. The second section discusses foods, food choices, and food shopping and is followed by a section on lifestyle stages—birth through aging. The last section is on “special issues,” and includes topics such as athletics, vegetarian eating, and allergies and a small section on managing common chronic diseases and supplement use.

Each chapter of the book focuses on the reader, labeled “you,” and the reader is challenged to become smarter about the topic presented and resultant behavior patterns. Food selection, food shopping, and home food storage issues are well covered, but no recipes are included. The book contains 676 pages of information, which is unique for food and nutrition guides aimed at the public.

I have seen this book grow and improve throughout its 3 editions, all of which have been popular with interested readers. The author has included the most recent governmental food guide information and nutrient recommendations. The writing style is very readable. The author uses clever phrases, such as “Carbs: Simply Complex,” “Sodium and Potassium: A Salty Subject,” and “Kitchen Nutrition: Delicious Decisions.”

I highly recommend this book as a ready reference for the person who is not a nutrition professional but who has a strong interest in following and knowing the latest on nutrition topics and recommendations for health.

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