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The satiating power of protein—a key to obesity prevention1,2

Arne Astrup

There is an urgent need for effective tools to prevent weight gain in the population at large and weight regain in overweight persons after weight loss. In theory the solution is simple, but implementation will continue to be difficult and ineffective as long as we maintain the view that just telling people that they should eat less and exercise more does the job. This simplistic strategy assumes that humans have conscious control over appetite and body weight regulation, which is certainly not the case for most people; if it were true, there would be no overweight or obese people. I have never met an obese patient who has worked hard to become obese and to maintain an excessive body size. We need to acknowledge that our regulatory systems are geared to prevent depletion of body energy stores and undernutrition effectively, whereas the systems that reduce appetite and increase energy expenditure during periods of excess availability of foods are easily suppressed by palatability and by the social, psychological, and rewarding aspects of foods.

In the past, when people expended plenty of calories just by going about their daily business, it was possible to remain slim while eating a diet with nearly any nutritional profile. The problem nowadays is that many people are extremely sedentary, which makes it possible to overeat even when dietary intakes are relatively small. For example, it is easy to prepare tasty, filling meals with drinks for an active person who needs relatively small. For example, it is easy to prepare tasty, filling meals with drinks for an active person who needs only 1800 kcal/d, but it is difficult to do the same for a sedentary person who needs only 1800 kcal/d. This is why the current focus of science is to increase the satiating power of the diet, so that people feel full with fewer calories.

An ad libitum diet that reduces the fat content from 40% to 25–30% of energy produces some weight loss (1). It is highly probable that the obesity rate would have increased even more over the past 20 y if the dietary fat content had not decreased during this period. However, because of the concomitant reductions in energy needs, increases in portion sizes, and increases in soft drink consumption that have occurred during the same period, the slight reduction in dietary fat over this time has clearly been insufficient to prevent weight gain and the increasing prevalence of obesity. The high obesity rate has also created a market for alternative, and often unsubstantiated, dietary solutions.

The higher than usually recommended protein content of many popular diets, such as the Atkins Diet, The Zone, and The South Beach Diet, seems to point at possible solutions to the obesity epidemic. Many national dietary guidelines have, until recently, recommended that only 10–20% of the calorie content of the diet come from protein; however, 30–40% of the calorie content in the aforementioned diets comes from protein, at the expense of carbohydrates. Newer research indicates that the high-protein content of these diets may actually be the reason for their partial success in inducing weight loss, despite no restrictions in total calories (2). High-protein diets, such as the Atkins diet, may also suppress food intake by producing ketosis. Ketosis results from the depletion of glycogen stores induced by a severe restriction of carbohydrates, to an extent that goes far beyond what is commensurate with a healthy diet.

In this issue of the Journal, Weigle et al (3) showed that an increase in dietary protein from 15% to 30% of energy and a reduction in fat from 35% to 20%, at a constant carbohydrate intake, produces a sustained decrease in ad libitum calorie intake and results in significant weight loss. They sequentially assigned 19 persons to the following diet regimens: 2 wk of a weight-maintenance diet (15% of energy as protein, 35% as fat, and 50% as carbohydrate), 2 wk of an isocaloric diet (30% of energy as protein, 20% as fat, and 50% as carbohydrate), or 12 wk of an ad libitum diet (30% of energy as protein, 20% as fat, and 50% as carbohydrate). They found that the subjects felt more satiated with the isocaloric high-protein diet than with the weight-maintenance diet. When the subjects were given the possibility to regulate their energy intake under the ad libitum conditions, spontaneous calorie intake decreased by 441 kcal/d, body weight decreased by 4.9 kg, and fat mass decreased by 3.7 kg. We do not yet understand how protein increases satiety. Weigle et al found that the effect could not be explained by changes in the hunger hormone ghrelin or in the satiety hormone leptin.

Weigle et al’s results clearly showed that protein is more satiating than is fat, and previous studies have shown that protein is more satiating than is carbohydrate (4). Moreover, diets with a fat content fixed at 30% of calories produce more weight loss when high in protein (25% of energy) than when normal in protein (12% of energy): 9.4 compared with 5.9 kg after 6 mo; after 1 y, evidence was found to suggest that the high-protein diet, independent of the loss of total body fat, resulted in a significant loss of visceral fat (5).

Should we advise overweight and obese patients to increase their protein intakes from 10–20% to 20–30% of calories and reduce their intake of fat and carbohydrates correspondingly? If

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Fat intake is fixed at ≈30% of calories, there is still plenty of room for carbohydrates to make up 40–50% of the calories. It is preferable to replace sugars from soft drinks with protein from low-fat milk, high-fat meat and dairy products with the lean versions, and possibly white bread and pasta with lean meat, without reducing the intakes of fruit, vegetables, and whole-grain products. Should we advise the public to increase their intakes of meat and dairy products? The answer depends on the potential adverse effects of a high-protein diet.

The guidelines from the Institute of Medicine allow for the inclusion of higher amounts of protein than previously recommended in a healthy diet (6). This institute concluded that there is no clear evidence that a high protein intake increases the risk of renal stones, osteoporosis, cancer, or cardiovascular disease. Thus, the acceptable protein distribution was set to 5–20% of calories for children aged 1–3 y, 10–30% for children aged 4–18 y, and 10–35% for adults. There is clearly a need for more long-term dietary intervention trials to address these issues, and the European Union has just funded (~18 million dollars) the DiOGenes (Diet, Obesity, and Genes) project, the core of which is a large multicenter trial of high- compared with low-protein diets and of high- compared with low-glycemic-index diets in obese and overweight families (7). This trial, which may involve participation by the United States, will investigate over 1 y the health effects of high-protein diets in 1500–2000 subjects. Perhaps now is the time to consider the economic and environmental consequences of increasing the population’s intake of protein from fish, meat, and vegetables and how this increase can be incorporated into the local cuisine.

AA is a member of several advisory boards for the food industry, is a Medical Advisor for Weight Watchers, and heads the dietary intervention study in the DIOGENES project (coordinated by WHM Saris, Netherlands); his department receives grants and food donations from ≈150 food companies.

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Alcohol, methylenetetrahydrofolate 677C→T genotype, and low folate intake: concurrent causes for hyperhomocysteinemia

Marilia Cravo

Homocysteine is a sulfur-containing amino acid that in recent years has become the subject of interest because of epidemiologic studies linking hyperhomocysteinemia with increased risk of occlusive vascular disease (1). Disposal of plasma or serum total homocysteine (tHcy) involves 2 metabolic pathways, remethylation and transsulfuration, which seem to be nutritionally regulated. The remethylation of tHcy into methionine requires folate and vitamin B-12, whereas vitamin B-6 is involved in tHcy removal through the transsulfuration pathway.

The possible link between tHcy and alcoholism stems from the fact that the abovementioned vitamins can be affected by chronic alcohol consumption (2). Hyperhomocysteinemia in chronic alcoholics was first reported by Hultberg et al (3), who found significantly higher concentrations of tHcy in a group of alcoholics hospitalized for detoxification than in a group of control subjects. Later, our group also confirmed that chronic alcoholics had tHcy concentrations twice those observed in nondrinkers, and we investigated further the correlation between folate, vitamin B-12, and vitamin B-6 status (4). Besides lower concentrations of these vitamins in chronic drinkers, we found a negative and significant correlation between plasma concentrations of tHcy and pyridoxal-5’-phosphate and a weaker negative correlation with red blood cell folates. These results strongly suggest that, by interfering with folate or vitamin B-6 metabolism, chronic alcohol abuse could impair the disposal of tHcy through the transsulfuration and transmethylation pathways, with the final result being a rise in tHcy concentrations. More recently, an animal study showed that alcohol ingestion, with or without folate deficiency, impairs the expression or activity (or both) of transmethylation enzymes that regulate homocysteine concentrations (5).

Since then, several studies have examined the relation between alcohol intake and plasma tHcy concentrations, but the results were rather contradictory (6–8). Whereas heavy alcohol abuse undoubtedly resulted in markedly elevated tHcy, the effect of moderate ethanol consumption on tHcy did not show a consistent pattern. These discrepancies may be related either to different methods of quantifying alcohol intake—ie, in grams per day rather than in drinks per month—or to the different types of alcoholic beverage consumed. Most studies are consistent with the observation that tHcy concentrations are significantly lower in beer drinkers (4, 6, 8) than in wine or spirits consumers. This difference may have to do with the fact that the amount of alcohol in beer is considerably lower (3.5%) than that in wine and spirits (12% and >35%, respectively) and also with the fact that beer is a rich source of folate and vitamin B-6, whereas wine and spirits contain negligible amounts of these vitamins (9). Consumption of beer in small amounts, despite its being an alcoholic beverage, could have a null or even a lowering effect on tHcy, as later confirmed by van der Gaag et al (10) in a prospective controlled study. This lowering effect on tHcy suggests a J-shaped effect, which is also consistent with the relations between alcohol consumption and the prevalence of cardiovascular disease, long known as the French paradox.

The article by Chiuve et al (11) in this issue of the Journal confirms the association between folate, ethanol, and tHcy and also extends our understanding of how the 677C→T polymorphism of methylenetetrahydrofolate reductase (MTHFR) may further modify the interaction between these variables. MTHFR is involved in the vitamin B-12–dependent methylation of homocysteine to methionine, and, in 1988, Kang et al (12) first reported a variant of MTHFR that was distinguished from the normal enzyme by its lower activity and its thermostability. This variant is caused by a point mutation (677C→T transition) at a polymorphic site that results in a valine substitution for an alanine. Heterozygotes (CT) and homozygotes (TT) have 71% and 33%, respectively, of the activity of the wild-type MTHFR (CC). The effect of the thermolabile variant of MTHFR on plasma homocysteine is now clear. Russo et al (13) showed that, when plasma folate concentrations were high, tHcy was low and unrelated to MTHFR genotype. However, when plasma folate concentrations were low, tHcy concentrations were significantly higher in carriers of the variant allele. The study by Chiuve et al was based on a sample of 988 premenopausal women with no history of cardiovascular disease who were part of the Nurses’ Health Study II cohort. The study aimed to examine whether the associations between folate intake and fasting plasma folate and tHcy concentrations were modified by MTHFR genotype or by alcohol intake. On the basis of self-reported alcohol use, women were divided into nondrinkers, those consuming <15 g alcohol/d, and those consuming >15 g alcohol/d. In secondary analyses, women who drank >50 g ethanol/d were excluded because

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heavy alcohol consumers may have dose responses between folate status and tHcy concentrations that are significantly different from those of moderate drinkers. In their cohort, Chiuve et al observed, consistent with previous studies, that tHcy concentrations decreased significantly with both folate intake and plasma folate concentrations and that the MTHFR polymorphism modified the dose-response association between folate intake and tHcy ($P$ for interaction = 0.05). Thus, women with the thermolabile variant of MTHFR677 (CT or TT) had significantly higher tHcy concentrations at low intakes of folate, whereas wild-type carriers had the smallest reduction in tHcy across extreme quintiles of folate intake. This finding reinforces the concept already discussed by others (12) that variant carriers are more sensitive to low folate intake, which raises the hypothesis that the protective effect of folate in regard to colorectal cancer or vascular disease is significantly more determinant in subjects carrying the polymorphic allele than in those carrying the nonpolymorphic allele.

With respect to alcohol intake, the authors observed that the variant or polymorphic allele modified the association between folate intake and tHcy ($P$ for interaction = 0.04), because higher folate intake was only modestly associated with lower tHcy among light drinkers (<15 g/d) and nondrinkers, whereas an inverse relation was significantly stronger among moderate drinkers (>15 g/d). As does the polymorphic allele, alcohol intake seems to exacerbate this modulating effect of folate status on tHcy concentrations. Furthermore, the inverse association between folate intake and tHcy among moderate alcohol drinkers was primarily limited to women with the variant allele for MTHFR, in whom there was a significant interaction between alcohol intake and genotype ($P$ = 0.01). To my knowledge, this is the first study in which the interactions between genotype, alcohol intake, and folate intake are examined together in a large group of apparently healthy persons. Although moderate alcohol consumers with the variant allele had high concentrations of tHcy at low folate intake, tHcy was no longer high in those with high folate intake. This was true only after the exclusion of heavy drinkers (>50 g/d) from the analysis, which highlights the fact that, whereas the adverse effects of moderate ethanol intake might be overcome through adequate folate supplementation, the same might not be true for the effects of heavy alcohol abuse.

We conclude that the elevation of tHcy among women who consume low amounts of folate and drink moderate amounts of alcohol seems to be further magnified in the presence of the variant MTHFR677 allele. This study adds further evidence that tHcy values must be interpreted in light of multiple variables such as age, sex, and smoking and drinking habits, among other lifestyle factors that may interact with each other as well as with several genetic traits of the methionine-homocysteine cycle, thereby influencing the final tHcy concentration.

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Interactive effects of iron and zinc on biochemical and functional outcomes in supplementation trials

Christa Fischer Walker, Katarzyna Kordas, Rebecca J Stoltzfus, and Robert E Black

ABSTRACT

Iron and zinc are essential micronutrients for human health. Deficiencies in these 2 nutrients remain a global problem, especially among women and children in developing countries. Supplementation with iron and zinc as single micronutrients enhances distinct and unique biochemical and functional outcomes. These micronutrients have the potential to interact when given together; thus, it is important to assess the biochemical and functional evidence from clinical trials before supplementation policies are established. We reviewed randomized trials that assessed the effects of iron and zinc supplementation on iron and zinc status. On the basis of this review, zinc supplementation alone does not appear to have a clinically important negative effect on iron status. However, when zinc is given with iron, iron indicators do not improve as greatly as when iron is given alone. In most of the studies, iron supplementation did not affect the biochemical status of zinc, but the data are not clear regarding morbidity outcomes. Although some trials have shown that joint iron and zinc supplementation has less of an effect on biochemical or functional outcomes than does supplementation with either mineral alone, there is no strong evidence to discourage joint supplementation. Supplementation programs that provide iron and zinc together are an efficient way to provide both micronutrients, provided the benefits of individual supplementation are not lost. Further research is needed before health policies on joint supplementation programs can be established. Am J Clin Nutr 2005;82:5–12.

KEY WORDS Micronutrients, zinc, iron, supplementation, women, children

INTRODUCTION

Iron and zinc are essential micronutrients for human growth, development, and maintenance of the immune system. Iron is needed for psychomotor development, maintenance of physical activity and work capacity, and resistance to infection (1). Zinc is needed for growth and for maintenance of immune function, which enhances both the prevention of and recovery from infectious diseases (2). Meat products are the best source of both iron and zinc. Consequently, iron and zinc deficiencies may coexist in populations that consume diets with insufficient amounts of animal-source foods. The intake of these 2 micronutrients would ideally be improved through enhanced dietary quality, but food fortification or supplementation programs may also be needed.

If iron and zinc are to be provided together, it is important to determine whether, and if so, how they interact biologically. Because they have chemically similar absorption and transport mechanisms, iron and zinc have been thought to compete for absorptive pathways (3). New evidence based on cell culture studies has shown that iron may inhibit zinc absorption in some cells at very high ratios of iron to zinc, but not vice versa (4). However, evidence of antagonism from studies of low ratios of iron to zinc is needed to assess any biochemical and functional effects of dual supplementation. Assessing the effect of single zinc or iron supplementation on the biochemical indicator of the other (ie, zinc on iron and iron on zinc) may help shed light on whether adverse effects are associated with supplementation with 1–2 times the recommended dietary allowance. Kordas and Stoltzfus (4) recently expounded on the gut interaction theory by reviewing new evidence for potential multiple interaction sites. Briefly, both iron and zinc are functionally important throughout the body and have the potential to interact in many systems, such as the nervous system. Although the separate functions of iron and zinc on brain and neural tissue are well described separately, studies of the possible interactions are scarce. Additional information to better understand the biological basis for potential interactions observed in functional outcomes such as growth, development, and disease resistance is needed.

The present review examines the evidence of iron and zinc interactions provided by placebo-controlled randomized trials of supplementation with iron or zinc alone or in combination in children aged <5 y and in women of child-bearing age. We addressed 4 distinct questions: 1) Does zinc supplementation alone affect iron status?, 2) Does iron supplementation alone affect zinc status?, 3) Does the joint supplementation of iron and zinc affect iron status? and 4) Does the joint supplementation of iron and zinc affect zinc status? If iron and zinc are provided together, it is important to determine whether, and if so, how they interact biologically.

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Does zinc supplementation alone affect iron status?

There are numerous iron-status indicators, such as hemoglobin, mean cell volume, erythrocyte protoporphyrin, serum iron, total-iron-binding capacity, transferrin saturation, serum ferritin, and serum transferrin receptor. Because many assessment techniques of varying sensitivity exist and because clinical trials often use a combination of different techniques to determine the effect of an intervention, it is often difficult to draw definitive conclusions on the basis of biochemical status alone.

In Nepal, 235 night-blind pregnant women were randomly assigned to receive vitamin A, β-carotene, or placebo and, in addition, daily zinc or placebo (15; Table 1). Zinc-supplemented women had a nonsignificant tendency for a greater decline in hemoglobin and a nonsignificant tendency for an increase in serum ferritin compared with women who received placebo. Osendarp et al (16) randomly assigned pregnant women to receive daily zinc or placebo. There was no difference in hemoglobin concentrations between zinc and placebo after 5 mo of supplementation.

Three studies were conducted in infants and none found effects of zinc supplementation on iron status. Dijkhuizen et al (17) randomized 371 Indonesian infants to receive iron, zinc, both iron and zinc, or placebo, 5 d/wk for 6 mo. After supplementation there were no differences in hemoglobin or serum ferritin concentrations between the zinc- or placebo-supplemented children. In another trial in Indonesia, 680 infants were randomly assigned to receive iron, zinc, both iron and zinc, or placebo daily for 6 mo (18). There were no differences in hemoglobin, serum ferritin, or serum transferrin receptor between children receiving zinc alone and children receiving placebo. Baqui et al (19) randomly assigned 799 Bangladeshi infants to receive iron, zinc, both iron and zinc, a multiple micronutrient mix (not reported here), or a control supplement weekly for 6 mo. The final hemoglobin concentration in the zinc-supplemented children was not significantly different from that in the control subjects after adjustment for age and baseline concentrations.

In Mexico, 219 toddlers were randomly assigned to receive iron, zinc, both iron and zinc, or placebo daily for 6 mo (20, 25). Zinc alone did not have a significant effect on hemoglobin or plasma ferritin concentrations compared with the placebo. Shankar et al (21) randomly assigned young children in Papua New Guinea to receive zinc or placebo daily for 5 mo. Although hemoglobin decreased in both groups, there was no difference between the zinc- and placebo-supplemented children. Children in Chile (22) were randomly assigned to receive zinc or placebo for 6 mo and no difference was observed between groups for either hemoglobin or serum ferritin. A small trial in Belize observed a higher hemoglobin concentration in children supplemented with zinc alone for 6 mo than in children given placebo after adjustment for the pretreatment means (23). Penny et al (24) randomly assigned Peruvian children with persistent diarrhea to receive zinc, multiple micronutrients, or placebo daily for 6 mo after the cessation of the episode. Although no difference in hemoglobin concentration was observed between the zinc- or placebo-supplemented children, there was a 8.1-μg/L increase in plasma ferritin in zinc-supplemented children compared with a 0.5-μg/L decrease in children given placebo ($P < 0.0001$).

In summary, in trials in which zinc was given at prophylactic doses to pregnant women and children aged <5 y, most of the trials showed no effect of zinc on hemoglobin or serum ferritin. Although one small trial showed a positive effect of zinc on hemoglobin and another trial showed a positive effect on plasma ferritin, it is most important to note that none of the trials showed a negative effect on iron indicators.
TABLE 1
Studies that assessed the effects of zinc supplementation on ion status

<table>
<thead>
<tr>
<th>Reference</th>
<th>Country</th>
<th>Enrollment criteria</th>
<th>Comparison groups</th>
<th>Duration of study</th>
<th>Iron indicators</th>
<th>Effect on iron status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Christian et al, 2001 (15)</td>
<td>Nepal</td>
<td>Pregnant women, night blindness</td>
<td>Vitamin A and B carotene or placebo and 25 mg Zn (n = 84) or placebo (n = 96)</td>
<td>3 wk</td>
<td>Hemoglobin, serum ferritin</td>
<td>No effect</td>
</tr>
<tr>
<td>Osendarp et al, 2000 (16)</td>
<td>Bangladesh</td>
<td>Pregnant women at 12–16 wk gestation</td>
<td>30 mg Zn (n = 194) and placebo (n = 216)</td>
<td>5 mo</td>
<td>Hemoglobin</td>
<td>No effect</td>
</tr>
<tr>
<td>Dijkhuizen et al, 2001 (17)</td>
<td>Indonesia</td>
<td>4-mo-old infants</td>
<td>10 mg Zn (n = 119) and placebo (n = 119)</td>
<td>6 mo</td>
<td>Hemoglobin, serum ferritin</td>
<td>No effect</td>
</tr>
<tr>
<td>Lind et al, 2003 (18)</td>
<td>Indonesia</td>
<td>6-mo-old infants</td>
<td>10 mg Zn (n = 134) and placebo (n = 143)</td>
<td>6 mo</td>
<td>Hemoglobin, serum ferritin</td>
<td>No effect</td>
</tr>
<tr>
<td>Baqui et al, 2003 (19)</td>
<td>Bangladesh</td>
<td>6-mo-old infants</td>
<td>1 mg Rb + 20 mg Zn (n = 161) and 1 mg Rb (control; n = 157)</td>
<td>6 mo</td>
<td>Hemoglobin, serum ferritin</td>
<td>No effect</td>
</tr>
<tr>
<td>Munoz et al, 2000 (20)</td>
<td>Mexico</td>
<td>18–36-mo-old children</td>
<td>20 mg Zn (n = 54) and placebo (n = 56)</td>
<td>6 mo</td>
<td>Hemoglobin, serum ferritin</td>
<td>No effect</td>
</tr>
<tr>
<td>Shankar et al, 2000 (21)</td>
<td>Papau New Guinea</td>
<td>6–60-mo-old children</td>
<td>10 mg Zn (n = 136) and placebo (n = 138)</td>
<td>5 mo</td>
<td>Hemoglobin</td>
<td>No effect</td>
</tr>
<tr>
<td>Ruiz et al, 1997 (22)</td>
<td>Chile</td>
<td>27–50-mo-old children</td>
<td>10 mg Zn (n = 49) and placebo (49)</td>
<td>6 mo</td>
<td>Hemoglobin, serum ferritin</td>
<td>No effect</td>
</tr>
<tr>
<td>Smith et al, 1999 (23)</td>
<td>Belize</td>
<td>22–66-mo-old children</td>
<td>70 mg Zn (n = 11) and placebo (n = 9)</td>
<td>6 mo</td>
<td>Hemoglobin</td>
<td>Positive effect of zinc on hemoglobin</td>
</tr>
<tr>
<td>Penny et al, 2004 (24)</td>
<td>Peru</td>
<td>6–35-mo-old children</td>
<td>10 mg Zn (n = 80) and placebo (79)</td>
<td>6 mo</td>
<td>Hemoglobin, serum ferritin</td>
<td>Positive effect of zinc on plasma ferritin</td>
</tr>
</tbody>
</table>

Does iron supplementation alone affect zinc status?

Most studies interested in zinc status use plasma or serum zinc as an indicator of outcome. It is important, however, to recognize the shortcomings of this indicator in reflecting zinc status when interpreting the effects of iron supplementation on zinc status. It may not be sensitive enough to detect antagonisms from iron supplementation, especially if these are relatively small in magnitude.

In the 4 trials in infants or toddlers mentioned previously (17–20), plasma zinc concentrations in children supplemented with iron alone did not differ from those in the control group (Table 2). In another study, 291 infants were randomly assigned to receive daily iron supplements or placebo (26). After 3 mo of supplementation, no difference in serum zinc concentration was observed between the iron and placebo groups. Friel et al (27) randomly assigned breastfed infants to receive iron or zinc for 5 mo and observed no difference in serum zinc concentrations between iron- and placebo-supplemented infants. In a multicountry trial of infant supplementation with multiple micronutrients, daily iron, or placebo, there was no difference between daily iron and placebo in 3 of the 4 study sites (28–30). However, in Indonesian infants, a greater percentage of infants who received daily iron (32.8%) than of those who received placebo (15.6%) were zinc deficient (<10.7 μmol/L) after 6 mo of supplementation (31). In this study there was no significant difference in the change in mean serum zinc after supplementation between the placebo- and iron-supplemented infants.

In 9 of 10 of these studies, iron supplementation did not have an effect on serum zinc status. It is not clear why the negative effect was seen in only one study. Although serum zinc is often criticized for being an unreliable measure of individual zinc status, on a population level the observed lack of effect of iron supplementation on this indicator may still be indicative of overall zinc status (32). These studies do not show evidence of adverse effects of iron on biochemical zinc status.

Does the addition of zinc to iron supplements affect iron status or functional outcomes?

Four trials assessed the effect of adding zinc to iron supplements in women and none found negative effects of combined zinc supplementation on iron status or anemia (Table 3). Kolskeren et al (33) randomly assigned 171 nonpregnant anemic women in Bangladesh to receive 1) iron, 2) iron and vitamin A, or 3) iron, vitamin A, and zinc; the results of a comparison of the groups that received the latter 2 treatments are reported here. Hemoglobin, serum ferritin, serum iron, percentage transferrin saturation (P < 0.0001 for all), and total-iron-binding capacity (P < 0.05) increased after 8 wk of daily supplementation with iron and vitamin A, with or without zinc. The women in this study were asked to take the iron and zinc supplements with separate meals; no attempt was made to ascertain whether the study participants complied with this request.

In Peru (5, 34, 35), 1295 pregnant women (33% anemic) were assigned to daily supplementation with iron and folic acid (IFA),...
with or without zinc. There were no differences between the groups supplemented with IFA alone or iron IFA and zinc on maternal hemoglobin (or proportion anemic) or serum ferritin concentrations at 28–30 or 37–38 wk of gestation or in cord blood hemoglobin or serum ferritin at delivery (35). There were no significant differences between the groups in length of gestation, fetal growth, or birth weight.

In the previously described trial in Nepal (15), night-blind pregnant women were also assessed for anemia and were supplemented with iron if needed. In women given IFA supplements, the addition of zinc increased maternal hemoglobin (or proportion anemic) and serum ferritin from baseline to the third trimester. The addition of iron and zinc supplements than after receiving iron alone (46% compared with 28%;  P < 0.05) (17). In another Indonesian study (18), supplementation with iron alone increased hemoglobin (119.4 compared with 115.3 g/L;  P < 0.05) and serum ferritin (46.5 compared with 32.3 μg/L;  P < 0.05) more than did supplementation with iron and zinc. The prevalence of anemia declined more in the iron-supplemented children than in the iron-and zinc-supplemented children ( P = 0.026); however, there was no difference in the prevalence of IDA between the 2 groups. In contrast, in Bangladeshi children (19), serum ferritin decreased in all groups after supplementation, but less so in the iron-and zinc-supplemented children (−13.7 μg/L;  P < 0.05 compared with control) than in the children supplemented with iron alone (−18.0 μg/L;  P < 0.3 compared with control).

In the previously described Mexican trial (20), hemoglobin concentrations increased in both the iron-only and iron- and zinc-supplemented groups by 14.0 g/L and 13.0 g/L, respectively, and the increases were significantly greater than in the placebo group (8.0 g/L;  P < 0.05). Both the iron-alone ( P < 0.05) and the iron plus zinc ( P < 0.0001) groups had significant increases in plasma ferritin concentrations from baseline to post follow-up. Schultink et al (39) randomly assigned 67 anemic Indonesian children to receive iron or iron plus zinc daily for 8

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### TABLE 2

<table>
<thead>
<tr>
<th>Reference</th>
<th>Country</th>
<th>Enrollment criteria</th>
<th>Comparison groups</th>
<th>Duration of study</th>
<th>Zinc indicators</th>
<th>Effect on zinc status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dijkstra et al, 2001</td>
<td>Indonesia</td>
<td>4-mo-old infants</td>
<td>10 mg Fe (n = 120) and placebo (n = 119)</td>
<td>6 mo</td>
<td>Serum zinc</td>
<td>No effect</td>
</tr>
<tr>
<td>Lind et al, 2003</td>
<td>Indonesia</td>
<td>6-mo-old infants</td>
<td>10 mg Fe (n = 136) and placebo (n = 143)</td>
<td>6 mo</td>
<td>Serum zinc</td>
<td>No effect</td>
</tr>
<tr>
<td>Baqui et al, 2003</td>
<td>Bangladesh</td>
<td>6-mo-old infants</td>
<td>1 mg Rb + 20 mg Fe (n = 165) and 1 mg Rb (control; n = 157)</td>
<td>6 mo</td>
<td>Serum zinc</td>
<td>No effect</td>
</tr>
<tr>
<td>Munoz et al, 2000</td>
<td>Mexico</td>
<td>18–36-mo-old children</td>
<td>20 mg Fe (n = 54) and placebo (n = 56)</td>
<td>6 mo</td>
<td>Serum zinc</td>
<td>No effect</td>
</tr>
<tr>
<td>Yip et al, 1985</td>
<td>United States</td>
<td>12-mo-old infants</td>
<td>30 mg Fe and placebo (n = 219)</td>
<td>3 mo</td>
<td>Serum zinc</td>
<td>No effect</td>
</tr>
<tr>
<td>Friel et al, 2003</td>
<td>Canada</td>
<td>1-mo-old term, breastfed infants</td>
<td>7.5 mg Fe (n = 19) and placebo (n = 19)</td>
<td>5 mo</td>
<td>Serum zinc</td>
<td>No effect</td>
</tr>
<tr>
<td>Smuts et al, 2005</td>
<td>South Africa</td>
<td>6–12-mo-old infants</td>
<td>10 mg Fe (n = 68) and placebo (n = 66)</td>
<td>6 mo</td>
<td>Serum zinc</td>
<td>No effect</td>
</tr>
<tr>
<td>Lopez de Romana et al,</td>
<td>Peru</td>
<td>6–12-mo-old infants</td>
<td>10 mg Fe (n = 82) and placebo (n = 82)</td>
<td>6 mo</td>
<td>Serum zinc</td>
<td>No effect</td>
</tr>
<tr>
<td>Hop et al, 2005</td>
<td>Vietnam</td>
<td>6–12-mo-old infants</td>
<td>10 mg Fe (n = 75) and placebo (n = 73)</td>
<td>6 mo</td>
<td>Serum zinc</td>
<td>No effect</td>
</tr>
<tr>
<td>Untoro et al, 2005</td>
<td>Indonesia</td>
<td>6–12-mo-old infants</td>
<td>10 mg Fe (n = 69) and placebo (n = 60)</td>
<td>6 mo</td>
<td>Serum zinc</td>
<td>Negative effect on the proportion of zinc-deficient subjects after supplementation</td>
</tr>
</tbody>
</table>

* Rb, riboflavin.
wk. Although both groups showed significant increases in hemoglobin after supplementation, the change in the iron-alone group (18 g/L) was significantly greater than the change in the iron and zinc group (8 g/L; \( P < 0.01 \)). Children supplemented with iron alone had a decrease in serum zinc after supplementation, whereas children receiving iron and zinc experienced an increase in serum zinc (\( P < 0.05 \); difference in changes).

In summary, there were no significant differences in outcome from supplementation with iron alone and with iron and zinc among pregnant women, but the results of supplementation with both iron and zinc in children were mixed. In some trials the expected improvements in iron-status indicators were not as great when zinc was added. Functional outcomes are far more important than are small biochemical differences, but data on these outcomes are limited. In one trial in pregnant women (37), IFA supplementation of pregnant women improved birth weight but IFA combined with zinc did not. The implications of this possible interaction are not yet understood.

**Does the addition of iron to zinc supplementation affect zinc status or functional outcomes?**

In 4 trials among infants and children, there were no adverse effects on plasma zinc concentrations of adding iron to zinc supplementation, and one trial suggested a benefit on morbidity (Table 4). In Indonesian infants (17) there was no difference in plasma zinc concentrations between children who received iron and zinc supplements and children who received zinc alone after 6 mo of supplementation. Growth was also assessed in this study, but no effect of supplementation was observed (17). In the other Indonesian trial (18), there was also no difference in serum zinc concentrations between the iron- and zinc-supplemented children and the children supplemented with zinc alone. In the Bangladeshi trial (19), serum zinc concentration improved in both the zinc-supplemented (0.08 mg/L; \( P < 0.01 \)) and the iron- and zinc-supplemented children (0.07 mg/L; \( P < 0.01 \)). The zinc plus iron group had a greater increase in serum zinc (\( P = 0.05 \) than

### Table 3
Studies that assessed the effects of the addition of zinc to iron supplementation on iron-status indicators and clinical outcomes

<table>
<thead>
<tr>
<th>Reference</th>
<th>Country</th>
<th>Enrollment criteria</th>
<th>Comparison groups</th>
<th>Duration of study</th>
<th>Iron indicators</th>
<th>Effect on iron indicators and clinical outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kolsteren et al, 1999 (33)</td>
<td>Bangladesh</td>
<td>15-45-y-old women, hemoglobin &lt;100 g/L</td>
<td>Vitamin A + 60 mg Fe (n = 57) and vitamin A + Fe + 15 mg Zn (n = 58)</td>
<td>60 d</td>
<td>Hemoglobin, ferritin, transferrin saturation, TIBC, serum iron</td>
<td>No effect</td>
</tr>
<tr>
<td>Caulfield et al, 1999 (34); Zavaleta et al, 2000 (35)</td>
<td>Peru Pregnant women, gestation between 10 and 24 wk</td>
<td>IFA [60 mg Fe + 250 μg folic acid (n = 495)] and IFA + 15 mg Zn (n = 521)</td>
<td>Until 4 wk postpartum</td>
<td></td>
<td>Hemoglobin, serum ferritin</td>
<td>No effect</td>
</tr>
<tr>
<td>Christian et al, 2001 (15)</td>
<td>Nepal Pregnant women, night blindness</td>
<td>Vitamin A and β-carotene or placebo plus 25 mg Zn + IFA [60 mg Fe + 400 μg folic acid (n = 64)] or placebo + IFA (n = 64)</td>
<td>3 wk</td>
<td></td>
<td>Hemoglobin</td>
<td>No effect</td>
</tr>
<tr>
<td>Christian et al, 2003 (36–38)</td>
<td>Nepal Newly pregnant women</td>
<td>IFA [60 mg Fe + 400 μg folic acid (n = 772)] and IFA + 30 mg Zn (n = 827)</td>
<td>Until 12 wk postpartum</td>
<td></td>
<td>Hemoglobin, serum ferritin, serum transferrin, serum iron</td>
<td>No effect on biochemical indicators</td>
</tr>
<tr>
<td>Dijkhuizen et al, 2001 (17)</td>
<td>Indonesia 4-mo-old infants</td>
<td>10 mg Fe (n = 120) and 10 mg Zn (n = 120)</td>
<td>6 mo</td>
<td></td>
<td>Hemoglobin, serum ferritin</td>
<td>No effect of zinc on hemoglobin or serum ferritin; antagonism of zinc on ability of iron to correct anemia</td>
</tr>
<tr>
<td>Lind et al, 2003 (18)</td>
<td>Indonesia 6-mo-old infants</td>
<td>10 mg Fe (n = 136) and 10 mg Zn (n = 136)</td>
<td>6 mo</td>
<td></td>
<td>Hemoglobin, serum ferritin</td>
<td>Smaller effect on hemoglobin, ferritin, and transferrin receptor than iron alone</td>
</tr>
<tr>
<td>Baqui et al, 2003 (19)</td>
<td>Bangladesh 6-mo-old infants</td>
<td>1 mg Rb + 20 mg Fe (n = 165) and 1 mg Rb + 20 mg Fe + 20 mg Zn (n = 162)</td>
<td>6 mo</td>
<td></td>
<td>Serum ferritin</td>
<td>Positive benefit of the addition of zinc on serum ferritin</td>
</tr>
<tr>
<td>Munoz et al, 2000 (20)</td>
<td>Mexico 18-36-mo-old children</td>
<td>20 mg Fe (n = 54) and 20 mg Fe + 20 mg Zn (n = 55)</td>
<td>6 mo</td>
<td></td>
<td>Hemoglobin, plasma ferritin</td>
<td>No effect</td>
</tr>
<tr>
<td>Schultink et al, 1997 (39)</td>
<td>Indonesia 30-50-mo-old children, &lt;−2 HAZ, hemoglobin &lt;110 g/L</td>
<td>30 mg Fe (n = 42) and 30 mg Fe + 30 mg Zn (n = 43)</td>
<td>8 wk</td>
<td></td>
<td>Hemoglobin</td>
<td>Antagonism of zinc on hemoglobin</td>
</tr>
</tbody>
</table>

\(^{1}\) TIBC, total-iron-binding capacity; IFA, iron and folic acid; Rb, riboflavin; HAZ, height-for-age z score.
did the control group, after adjustment for age and baseline concentrations. The iron- and zinc-supplemented infants had a 19% lower relative risk of severe diarrhea than did the control infants \((P < 0.05)\) during the 6 mo of the study. Zinc alone resulted in a nonsignificant 2% decreased relative risk of severe diarrhea. When malnourished infants \(< -1 \text{ weight-for-age } z\) score\) were examined separately, those who were supplemented with iron and zinc had a 30% lower risk of severe diarrhea \((P < 0.01)\) and a 40% lower risk of severe acute lower respiratory tract infections \((P < 0.05)\) than did the control group.

In a previously described population of Mexican toddlers (20), zinc concentrations increased in both zinc groups with and without iron and were not significantly different from each other. The zinc-supplemented groups (with or without iron) had significantly fewer total illness episodes and diarrhea episodes per child than did the placebo group (25). The coadministration of iron and zinc did not reduce the benefit of zinc.

Iron supplementation, in combination with zinc supplementation, does not appear to have an effect on serum zinc concentrations, but only 2 of the studies reviewed considered morbidity outcomes—an important indicator of subclinical zinc deficiency in populations with low-zinc diets (32). Because the success of zinc-supplementation programs would be measured in improvements in morbidity indicators, not serum zinc status, these clinical outcomes are of great importance.

### Evidence assessing joint supplementation on growth and development indicators

Iron and zinc are both important components of neural function and are essential for childhood growth and development (4). One study assessed the effect of zinc, iron, or both on infant development. The previously described Bangladeshi infants were also assessed for developmental outcomes (19, 40). At follow-up, the iron- and zinc-supplemented group had a significantly smaller decrease in the Psychomotor Development Index \((PDI)\) than did the control group. The PDI is a motor development score obtained from the Bayley Scales of Infant Development II; higher scores indicate a better outcome. There were no differences in PDI scores between the supplementation groups.

Lind et al (41) assessed 650 Indonesian infants supplemented daily with iron, zinc, both iron and zinc, or placebo. Zinc alone increased weight-for-age \(z\) scores and knee-heel length. Iron alone increased knee-heel length. In addition, PDI also improved in the iron-only group compared with the placebo group. Joint supplementation had no effect on either growth or developmental outcomes. Growth was also assessed in the previously described supplementation trial of Mexican children (25). After 12 mo of supplementation with iron, zinc, both iron and zinc, or placebo, growth was not significantly different between supplementation groups.

### SUMMARY AND CONCLUSIONS

Supplementation with multiple micronutrients would be an appealing strategy for the prevention and treatment of anemia and common morbidities that affect women and young children. However, drawing definitive conclusions regarding the potential benefit or harm of joint supplementation, based on a variety of study designs, target populations, and outcome measures has proven challenging.

This review found that joint supplementation generally does not negatively affect the biochemical outcomes expected from individual supplementation. Three of 9 trials (Table 3) found that zinc may reduce the beneficial effect of iron supplements on iron status, but this negative interaction does not appear to be great enough to discourage joint supplementation. Even in the presence of zinc, the benefit of iron supplementation on iron indicators was significant and important. Iron does not appear to have a negative effect on serum zinc concentrations; if there is an effect, it is small.

Limited data exist on the effect of dual supplementation on infectious disease morbidity, growth, and child development. Many trials have shown a reduction in diarrhea and pneumonia morbidity with zinc supplementation (13, 42). Whereas one study showed that supplementation with both zinc and iron had a slightly greater benefit on diarrhea and respiratory morbidity than did zinc alone (19), more evidence is needed to confirm that the concurrent provision of iron does not reduce the benefits of zinc on infectious morbidity. Finally, additional information is needed to understand the effect of joint zinc and iron supplementation on growth and development.

The currently available data do not allow for firm conclusions on the existence of interactions between iron and zinc, when given together, on biochemical or functional outcomes. Clear programmatic recommendations cannot be made without further studies. Trials in nonanemic pregnant women should be done to ensure that the most beneficial combination of iron and zinc supplementation is promoted to ensure adequate stores before
pregnancy. Trials in pregnant women are needed to determine whether zinc supplements provide benefits to the mother or infant and to verify that the addition of iron to maternal iron supplements will not diminish the benefits of iron on anemia or birth weight.

Additional trials in children aged < 5 y are needed to ensure that the addition of iron to zinc supplements will not diminish the benefits of zinc supplementation, namely reductions in diarrhea, pneumonia, and other morbidities. Because the iron-regulatory mechanisms of infants may differ before and after 9 mo of age, studies in both of these age groups are needed to understand more fully the health effects of micronutrient supplementation. Finally, limited data suggest that supplementation with both iron and zinc may prevent developmental delays that may otherwise occur in the first year of life in vulnerable populations. Future studies need to assess the effect of joint supplementation on growth and child development and not just the global measures of cognitive functions to more specific developmental outcomes, for which the benefits of treatment and interactions might be more easily observed.

Thus far, iron and zinc interaction studies have focused on the effects of antagonisms on biochemical indicators. Although these effects should be measured in future trials, clinically meaningful health outcomes in mothers and infants will provide more useful information than will biochemical outcomes alone.

REB and RJS were responsible for the study concept. CFW was responsible for the literature research and the initial draft preparation. KK, REB, RJS, and CFW were responsible for the draft and revision of the manuscript. None of the authors had personal or financial conflicts of interest with regard to the review.

REFERENCES

Association of physical activity with body-composition indexes in children aged 6–8 y at varied risk of obesity1–3


ABSTRACT

Background: Physical inactivity increases the risk of obesity, but the relations between reported levels of physical activity (PA) and measures of body fatness (BF) in children are remarkably inconsistent.

Objective: We examined the relation between objective measures of PA and body-composition indexes in nonobese children.

Design: A cross-sectional study was conducted in 100 children aged 6–8 y who were recruited according to their risk of future obesity: high-risk children had ≥1 obese parent [body mass index (BMI; in kg/m²): >30] and low-risk children had 2 nonobese biological parents (BMI: <30). Free-living activity energy expenditure (AEE) and PA level were calculated from 7-d doubly labeled water measurements, time spent in light-intensity activity was assessed by heart rate monitoring, and body composition was determined from isotopic dilution. To adjust for body size, fat mass and fat-free mass were normalized for height and expressed as fat mass index (FMI) and lean mass index (LMI), respectively.

Results: High-risk children had significantly higher BMI, LMI, and FMI than did low-risk children, but no group differences in PA were found. AEE and PA level were positively associated with LMI and, after adjustment for sex and fat-free mass, negatively associated with FMI but not with BMI. Boys who spent more than the median time in light-intensity activities had significantly higher FMI than did less sedentary boys. This difference was not observed in girls.

Conclusions: AEE and PA level were negatively associated with BF in nonobese children. Accurate measures of body composition are essential to appropriate assessment of relations between PA and obesity risk.

KEY WORDS Children, physical activity, energy expenditure, body composition, obesity

INTRODUCTION

The prevalence of childhood obesity continues to increase, and established obesity is difficult to resolve. Therefore, an urgent need exists to identify modifiable risk factors for obesity to mitigate the emerging epidemic. It is now well established that children with ≥1 obese parent are at high risk of excess weight gain in both childhood and adolescence and that the risk increases with age (1, 2). However, it is not clear which environmental factors, in particular which components of physical activity and diet, are the most important contributors to this risk.

Although the increase in childhood obesity is frequently attributed to a decline in physical activity (PA), a remarkable lack of consistency exists in the relations between reported levels of PA and degrees of fatness (3, 4). This inconsistency could be due to methodologic flaws in assessing activity, inactivity, and body composition. The cross-sectional nature of many of the studies that have used objective measures of activity in children and adolescents to compare activity levels in lean and obese children do not allow the establishment of cause-and-effect relations (5–11). Studies in healthy children that examined relations between activity measures–derived estimates of energy expenditure from doubly labeled water (DLW) and measures of fatness reported mixed results; some reported a negative relation (7, 12–16), and others reported no relation (17–19). Furthermore, few studies have examined associations between objective measures of the intensity of activity and fatness. One study that used heart rate...
(HR) monitoring in 28 boys aged 9 y found a positive association between time spent on sedentary activities and percentage body fat (%BF) but not between activity and %BF (20), whereas a relation between vigorous activity, assessed by accelerometry, and %BF was observed in studies of prepubescent children (16, 21, 22). However, no studies have examined associations between PA and fatness in lean children at different risks of obesity.

This study was designed to establish the relation between PA and body composition in nonobese children by simultaneously using 2 independent and objective measures of PA and by using an approach to optimally normalize body-composition variables for body size. Furthermore, we sought to determine in healthy nonobese children, some of whom could be considered preobese on the basis of parental characteristics, whether different associations were apparent between PA, activity energy expenditure (AEE), and sensitive measures of body composition in prepubescence.

SUBJECTS AND METHODS

Subjects

One hundred healthy children (60 boys, 40 girls) participated. They were recruited into 2 groups according to their risk (high or low) of future obesity (Table 1). High-risk children had ≥1 biological parent with a body mass index (BMI; in kg/m²) ≥30, and low-risk children had 2 nonobese biological parents (BMI < 30).

The children were recruited from schools in the Coleraine area of Northern Ireland (United Kingdom). The town of Coleraine has a mixed socioeconomic background, and the population of ≈ 55,000 is predominantly white European. Parents of eligible children (ie, those aged 6–8 y and living with biological parents) were first contacted by letter, after which those who expressed interest in participating in the study were interviewed in their homes to explain the study in detail. Parents were informed that the study concerned the measurement of energy expenditure and food intake of children. No direct reference was made to obesity because this could have biased the recruitment of subjects. All measurements took place over the school term and were conducted during the school term and were conducted over a 3-y period.

The parents of each subject gave written informed consent to their child’s participation in the study, and no subject who agreed to participate was subsequently excluded. The Ethics Committee of the University of Ulster approved the study.

Anthropometry

In the metabolic suite at the University of Ulster, body weight in a swimsuit was measured to the nearest 0.1 kg (Weylux Model 824/890; CMS Weighing Equipment, London, United Kingdom), and height was measured to the nearest 0.1 cm with the use of a stadiometer (CMS Weighing Equipment) and standardized procedures. The weight and height of both parents, in light clothing and not wearing shoes, were also measured under standardized conditions. BMI was calculated for the children and the parents. For the children, the international reference standard was used to define overweight and obesity (23). This standard is based on average centiles, which equate to a BMI of 25 or 30 at age 18 y for overweight or obesity, respectively. Here, obesity is defined as a BMI on or above the obesity cutoff, whereas overweight is a BMI on or above the overweight cutoff and below the obesity cutoff. Body fat mass in children was measured by isotopic dilution during the measurements of energy expenditure by using the DLW method.

Body composition and activity energy expenditure

Total energy expenditure (TEE) was measured over 10 d by the DLW method. After collection of a predose urine sample, every child was given oral doses of 0.05 g 2H₂O/kg body wt and 0.125 g H₁₈O/kg body wt. Further samples were collected 8 h after dose and, thereafter, at a known time daily for 10 d. Samples were stored at −4 °C before measurement. A detailed description of the measurement was previously published (24).

Besides being used to calculate TEE, the intercepts of the isotope disappearance curves were used to provide estimates of total body water. Body water was calculated as the mean of the time zero H₂O distribution/1.01 and of the time zero ₂H₂O distribution space/1.04. Fat-free mass (FFM) was calculated from total body water by dividing the water content of fat-free tissue with age- and sex-specific values (25). Fat mass (FM) was

### Table 1

<table>
<thead>
<tr>
<th>Physical characteristics of the participants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Boys</strong></td>
</tr>
<tr>
<td><strong>Low-risk</strong> (n = 29)</td>
</tr>
<tr>
<td>Age (y)</td>
</tr>
<tr>
<td>Weight (kg)</td>
</tr>
<tr>
<td>Height (m)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>Overweight [% (%)]²</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
</tr>
<tr>
<td>Fat mass index (kg/m²)</td>
</tr>
<tr>
<td>Lean mass index (kg/m²)</td>
</tr>
<tr>
<td>Percentage body fat [%]</td>
</tr>
<tr>
<td>Mother’s BMI (kg/m²)</td>
</tr>
<tr>
<td>Father’s BMI (kg/m²)</td>
</tr>
</tbody>
</table>

¹ There were no significant interactions between sex and risk group by two-factor ANOVA.
² ± SD (all such values).
³ Determined by using International Obesity Task Force BMI cutoffs (23); chi-square test for overweight.
calculated as the difference between body weight and FFM. For comparison between subjects, FM and FFM were divided by height squared and expressed as FM index (FMI) and lean mass index (LMI) to adjust for body size (26, 27).

It is difficult to measure basal metabolic rate (BMR) in young children, because they are often unable to lie at rest for the prolonged periods needed to make accurate measurements. Therefore, we used predictive equations based on the weight, height, sex, and age of the child to estimate BMR; these equations were previously shown to have good agreement with measured BMR (28–30). AEE was calculated as

\[ (0.9 \text{ TEE}) - \text{BMR} \]  

which included a correction for 10% thermogenesis (31), and PAL was also calculated (TEE/BMR). Estimated energy requirements were calculated from published sex- and age-specific equations that are based on height and weight (32). These equations, derived from collated DLW energy expenditure data, allow for 4 levels of activity—sedentary, low activity, active, and very active—and for a corresponding activity coefficient in the energy equations. Estimated energy requirements were calculated for the low activity and active levels.

Heart rate monitoring

During the DLW study period, patterns of free-living physical activity were also assessed by HR monitoring (Polar PE3000 monitor; Polar Electro, Kempele, Finland). The objective was to obtain 7-d (daytime only) HR data, which were representative of all the days of the week. The subjects were asked to wear the monitor at all times except while swimming or showering (the monitors were not waterproof). At the end of the study period, minute-by-minute HR data were downloaded into a personal computer with the use of an interface.

Before the monitoring of HR under free-living conditions, maximal HR was measured on a motorized treadmill by using an incremental exercise protocol (33). During the test, the children ran for 3-min periods at increased workloads until peak HR was reached at volitional exhaustion. Good agreement was observed, and there were no significant differences between the measured peak HR and estimated peak HR from published equations (34).

The percentage of time spent in light-intensity activity and vigorous-intensity activity (vigorous activity) was estimated as follows (35):

\[ \text{Time} \leq \text{child's 50\% peak HR/total HR recorded time} \]

for each day \( \times 100 \)  

and

\[ \text{Time} \geq \text{child's 70\% peak HR/total HR recorded time} \]

for each day \( \times 100 \)

In addition, the light-intensity activity and vigorous activity of the children were further categorized into low and high levels of activity according to the amount of time spent below and above the median time, respectively. For light-intensity activity, this value was 36.6%, and that for vigorous activity was 6.6%.

Statistical analysis

Results are expressed as mean ± SD; values for nonparametric variables, which could not be normalized by transformation, are expressed as medians and interquartile ranges (IQRs). Differences between sexes and risk groups and the PA categories were assessed by using \( t \) tests and the nonparametric Mann-Whitney \( U \) test, as appropriate. Two-factor analysis of variance was used to test for sex \( \times \) risk group interactions for the physical characteristics of the participants. In nonparametric variables, the two-factor analysis of variance of the ranks was undertaken, and, if significant, the Kruskal-Wallis test was used to compare the subgroups. Wilcoxon’s signed rank test was undertaken to assess possible differences between time spent in light-intensity activities and vigorous activities on weekdays and weekends and differences in measured TEE and estimated energy requirements. Correlations between AEE, PAL, and time spent in light-intensity and vigorous activities were examined with Spearman’s.

Multiple linear regression analysis was used to examine associations of body-composition indexes with AEE and PAL. AEE and PAL were log-transformed by using natural logs. Because FFM is an important predictor of energy expenditure (31), models exploring the association between AEE and FMI were further adjusted for FFM (in kg) as a covariate. Although, in the case of BMI, FFM is a component of weight, the inclusion of FFM as a confounder in the final model is informative. To ensure that adequate adjustment for variation in body size was made, relations between FM and height and between FFM and height were also assessed by using log-log regression analysis. The \( \beta \)-coefficient of logged height corresponds to the power \( P \) by which height should be raised to calculate an index for FFM and FM, which is uncorrelated with height. This method for normalizing for body size has been described in detail elsewhere (36). This method was also used to normalize AEE for FFM. Values of \( P < 0.05 \) were regarded as significant, and all statistical analyses were performed with the use of SPSS software (version 11.0; SPSS Inc, Chicago, IL).

RESULTS

The physical characteristics of the study participants are presented in Table 1. No significant interactions between sex and risk group were found. Children in the high-risk group (31 boys, 19 girls) had significantly greater weight (\( P < 0.05 \)), BMI (\( P < 0.01 \)), waist circumference (\( P < 0.05 \)), %BF (\( P < 0.05 \)), LMI (\( P < 0.01 \)), and FMI (\( P < 0.05 \)) than did children in the low-risk group (29 boys, 21 girls), but the former group did not differ significantly in age or height. In the high-risk group, 44% of fathers and 46% of mothers were classified as obese, whereas 10% of the high-risk children had both parents classified as obese. With the use of the International Obesity Task Force BMI cutoffs, none of the children were defined as obese, but significantly (\( P < 0.01 \)) more children in the high-risk group (\( n = 11 \)) than in the low-risk group (\( n = 1 \)) were classified as overweight. Boys were significantly taller and had a significantly higher LMI than girls (\( P < 0.05 \)).

Physical activity intensity levels and patterning

Complete HR data were collected from 42 boys and 19 girls for a median of 8.75 h/d for a median of 6 d. No differences were
observed in the body-composition characteristics of those who completed HR monitoring and those who did not, except that the girls who completed HR monitoring had a significantly ($P/L_{0.009}$) higher FMI.

Activity energy expenditure and physical activity

On all tests of sex × risk group interactions for measures of energy expenditure and PA ($P/L_{0.05}$), TEE and PAL were significantly higher in boys than in girls ($P/L_{0.01}$). Although TEE was significantly higher in the high-risk group than in the low-risk group ($P/L_{0.05}$), no differences in PAL were observed between the risk groups (Table 2). With a median of 388 kJ/d (IQR: 977, 79 kJ/d), TEE was significantly ($P/L_{0.001}$) lower than the estimated energy requirements for an active lifestyle (31) but significantly ($P/L_{0.001}$) higher than the estimate for low activity by 570 kJ/d (IQR: 58, 1117 kJ/d); however, this difference was not attributable to differences between the sexes or between the risk groups.

Boys expended significantly more energy in activity than did girls, and 43% of the difference in AEE between the sexes was accounted for by FFM. No difference in AEE or PAL between the risk groups was found when adjusted for sex or when further adjusted for FFM.

No significant differences were observed between boys and girls or between the risk groups in either the median or IQR total amounts of time spent in light-intensity activity (boys: 25.9%; IQR: 11.1–55.8%; girls: 31.9%; IQR: 20.9–54.7%) (Table 2) or between the time spent in light-intensity activity on weekends and on weekdays. Although time spent in vigorous activities did not differ significantly between risk groups, the boys spent significantly more time overall in vigorous activities during the week (median: 6.1%; IQR: 3.2–9.7) than on the weekends (median: 1.0%; IQR: 0–4.1; $P/L_{0.001}$). No differences in vigorous activities between weekdays and weekends were observed in girls.

PAL was not associated with time spent in either vigorous or light-intensity activity, nor was AEE associated with time spent in vigorous activity in boys or girls. Although AEE was positively associated with time spent in light-intensity activity

| TABLE 2 | Measures of energy expenditure and physical activity in prepubertal children by sex and risk group$^1$ |
|---------|---------------------------------|-----------------|---|-----------------|
|         | Boys                            | Girls           |     | Significant main effects |
|         | Low-risk ($n = 29$)             | High-risk ($n = 31$) |     | Low-risk ($n = 21$) | High-risk ($n = 19$) |     |
| TEE (MJ/d)$^2$ | 7.79 ± 1.1$^2$ | 8.11 ± 1.2 | 6.44 ± 0.68 | 7.14 ± 0.81 | Sex, Risk |
| AEE (MJ/d)    | 2.58 ± 0.73 | 2.74 ± 0.79 | 1.82 ± 0.51 | 2.27 ± 0.65 | Sex |
| PAL (TEE/BMR) | 1.76 ± 0.16 | 1.77 ± 0.17 | 1.62 ± 0.14 | 1.72 ± 0.18 | Sex |
| Vigorous activity (% time $>70\%$ peak HR)$^3$ | 8.9 (4.1, 13.1) [20] | 6.9 (4.6, 12.3) [22] | 6.5 (4.2, 8.0) [10] | 5.1 (4.0, 18.0) [9] | — |
| Light-intensity activity (% time $<50\%$ peak HR)$^4,5$ | 33.6 (15.6, 54.0) [20] | 38.9 (27.2, 57.9) [22] | 36.2 (28.6, 58.3) [10] | 26.5 (13.9, 67.9) [9] | — |

$^1$ TEE, total energy expenditure; AEE, activity energy expenditure; PAL, physical activity level; BMR, basal metabolic rate; HR, heart rate. There were no significant interactions between sex and risk group by two-factor ANOVA.

$^2$ ± SD (all such values).

$^3$ (0.9 TEE) − BMR.

$^4$ Median; interquartile ranges in parentheses (Mann-Whitney U tests); $n$ for risk group in brackets.


**FIGURE 1.** Activity energy expenditure (AEE) calculated from doubly labeled water data for total energy expenditure and estimated basal metabolic rate and time spent in light-intensity activity (in %) in 100 prepubertal boys (C) and girls (□) [(A) $r = 0.32$, $P = 0.01$] and AEE adjusted for fat-free mass (in kg) [(B) $r = 0.09$, NS]. AEE was normalized for fat-free mass (AEE/fat-free mass$^{1.3}$).
(Spearman’s $\hat{\rho} = 0.32, P = 0.01$), this association was no longer significant after AEE was normalized for fat-free body mass (Spearman’s $\hat{\rho} = 0.09$) (Figure 1).

**Physical activity and body composition**

Boys who spent more than the median time in light-intensity activity ($\geq 36.6\%$) had significantly higher FMI than did boys who spent less than the median time in light-intensity activities ($P = 0.04$ for sex $\times$ light-intensity activity category interaction) (Table 3). However, this association was not observed in girls. No associations between vigorous activity category (median: $<6.6\%$ and $\geq 6.6\%$ of time per day) and body composition were found.

Both AEE and PAL were significantly ($P < 0.001$) positively associated with FFM after adjustment for sex ($\beta$-coefficient: 5.88 and 11.5; SE: 0.89 and 3.26, respectively) and were negatively associated with FMI after adjustment of the latter for sex and fat-free mass ($\beta$-coefficient: $-1.41$ and $-5.25$; SE: 0.44 and 1.19; $P = 0.002$ and $< 0.001$, respectively). The associations of AEE and PAL with FMI were further strengthened after adjustment for risk group ($\beta$-coefficient: $-1.53$ and $-5.57$; SE: 0.43 and 1.16; $P = 0.001$ and $< 0.001$, respectively) (Table 4). The final AEE model adjusted for sex, FFM, and risk group explained 17% of the variance in FMI; AEE explained 59% of this variance (Figure 2). The final PAL model explained 24% of the variance in FMI, and PAL itself explained 71% of the variance (Figure 3). All analyses were repeated with FM and FFM that were normalized with the use of more specific powers for height obtained with the use of log-log regression analysis ($P = 2.9$ for FM and $P = 2.2$ for FFM). All the relations observed with the body-composition indexes remained essentially unchanged. These results are reproducible when weight is used instead of FFM as a covariate in the analyses.

A higher BMI was significantly associated with higher AEE ($\beta$-coefficient: 1.27; SE: 0.57; $P = 0.02$), but this association was attenuated, becoming nonsignificant and negative after control for FFM ($\beta$-coefficient: $-0.69$; SE: 0.58). No association of BMI with PAL was observed, with or without adjustment for FFM.

**DISCUSSION**

In this study, we measured activity by 2 separate and objective methods that allowed both the accurate quantification of energy expenditure (ie, DLW) and the patterning and intensity of activity (ie, HR monitoring). In both boys and girls, consistent negative associations were observed between FMI and AEE and PAL. In addition, in boys, negative associations between a measure of fatness and light-intensity activity were found. TEE was significantly lower (5%) than the estimated energy requirements for children with an active lifestyle but significantly higher (8%).

---

**TABLE 3**

<table>
<thead>
<tr>
<th>Light-intensity activity</th>
<th>Boys</th>
<th></th>
<th>Girls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low activity</td>
<td>High activity</td>
<td>Low activity</td>
<td>High activity</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>22</td>
<td>11</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>15.3 (14.6–16.4)</td>
<td>16.9 (15.7–17.8)</td>
<td>15.8 (14.8–17.5)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>55.4 (54.0–57.9)</td>
<td>61.0 (56.7–64.6)</td>
<td>57.8 (53.9–59.2)</td>
</tr>
<tr>
<td>Fat mass index (kg/m²)</td>
<td>1.03 (0.54–1.82)</td>
<td>1.89 (1.11–3.02)d</td>
<td>2.75 (1.57–3.48)</td>
</tr>
<tr>
<td>Lean mass index (kg/m²)</td>
<td>14.2 (13.5–14.8)</td>
<td>14.7 (13.9–15.1)</td>
<td>13.3 (12.5–14.6)</td>
</tr>
</tbody>
</table>

Vigorous activity

<table>
<thead>
<tr>
<th>n</th>
<th>19</th>
<th>23</th>
<th>12</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>15.9 (15.0–17.5)</td>
<td>15.8 (14.5–17.4)</td>
<td>17.1 (14.8–18.1)</td>
<td>15.8 (13.7–16.5)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>60.2 (55.0–64.6)</td>
<td>56.7 (53.6–60.3)</td>
<td>57.6 (54.7–61.2)</td>
<td>57.8 (49.1–59.2)</td>
</tr>
<tr>
<td>Fat mass index (kg/m²)</td>
<td>1.47 (0.89–2.05)</td>
<td>1.41 (0.61–2.28)</td>
<td>2.61 (1.33–3.30)</td>
<td>2.68 (1.60–3.62)</td>
</tr>
<tr>
<td>Lean mass index (kg/m²)</td>
<td>14.5 (13.8–14.8)</td>
<td>14.5 (13.7–15.1)</td>
<td>14.3 (13.1–14.7)</td>
<td>12.5 (11.8–13.7)</td>
</tr>
</tbody>
</table>

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1 All values (except n) are medians; interquartile ranges in parentheses. There were no significant main effects of sex or risk and no interactions between sex and risk group by two-factor ANOVA, except where indicated.

2 Time spent in light-intensity activity: low, $<36.6\%$; high, $\geq36.6\%$.

3 $P$ for interaction = 0.4.

4 $P = 0.03$ for fat mass index between light-intensity activity categories in boys (Kruskal-Wallis test).

5 Time spent in vigorous activity: low, $<6.6\%$; high, $\geq6.6\%$.

---

**TABLE 4**

Regression analyses of energy expenditure measures of activity with fat mass index models

<table>
<thead>
<tr>
<th>Sex</th>
<th>Risk group</th>
<th>Fat-free mass</th>
<th>AEE$^2$</th>
<th>PAL$^2$</th>
<th>$R^2$</th>
<th>$P$ for model</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEE model</td>
<td>0.25 (0.24)</td>
<td>0.52 (0.22)</td>
<td>0.14$^*$ (0.04)</td>
<td>$-1.53^*$ (0.43)</td>
<td>0.17</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>PAL model</td>
<td>0.30 (0.22)</td>
<td>0.53$^*$ (0.21)</td>
<td>0.12$^*$ (0.03)</td>
<td>$-5.57^*$ (1.16)</td>
<td>0.24</td>
<td>$&lt;0.001$</td>
</tr>
</tbody>
</table>

$^1$ All data (except $P$ values) are $\beta$-coefficients; SEs in parentheses.

$^2$ Log, transformed.

$^3$ $P < 0.05$.

$^4$ $P < 0.01$. 

---
than the estimated requirements for children with low activity levels. Two other studies reported that mean TEE in prepubescent children is 9–13% lower than estimated energy requirements (13, 37). However, both of those studies used the older (1985) FAO/WHO/UNU recommendations for comparison, and those recommendations were based on reported dietary intakes rather than on DLW measures of TEE (38).

Children are at increased risk of future obesity when they have parents who are obese (1). We have shown that, even in nonobese prepubescent children, consistent body-composition differences exist between those with ≥1 obese parent and those with nonobese parents. In our study, significantly higher BMI, FMI, and LMI and significantly larger waist circumferences were found in the high-risk group than in the low-risk group. In a smaller study of 49 children aged 6–10 y, those with an obese mother had significantly higher tissue mass in boys, but no associations with body composition and overweight children.

Similarly, in examinations of associations between activity and FM, adjustment for FFM is critical to preventing inappropriate conclusions. Interindividual variability in FFM is important, because, after adjustment for size, children vary substantially in FFM as well as in fatness (44). In the current study, no association was found between AEE and FMI before adjustment for FFM. In the case of BMI, if we had not adjusted AEE for FFM, we would have concluded from our data that there was a significant positive association with AEE. However, after that adjustment, the opposite conclusion was justified. This illustrates the real dilemma in interpreting BMI as a measure of body fatness, without proper recognition that weight is a mixed measure of FFM and FM.

The association between PAL and FMI was stronger than observed between AEE and FMI, after adjustment for sex, risk group, and FFM. Some (7, 12–16) but not all (17–19) studies have reported cross-sectional inverse associations between PAL and body fat. In a prospective study, Goran et al (41) found no inverse relation between AEE and change in FM, after adjustment for FFM, over a 4-y period in prepubescent children. These discrepant results could reflect real differences between populations, but, again, they are more likely to be a consequence of the different approaches used for normalizing body size. For example, when using PAL, it is often assumed that the potential influence of body size is resolved by adjustment for BMR, but, in our study, PAL was found not to be independent of body size. In fact, it was significantly correlated with both body weight and FFM in boys and girls (weight: \( r = 0.52 \) and 0.35; \( P < 0.001 \) and 0.03; FFM: \( r = 0.59 \) and 0.46; \( P < 0.001 \) and 0.001, respectively). However, after adjustment for fat-free mass, the association between PAL and FMI was stronger (\( \beta \)-coefficient: \( -2.16 \) and \( -3.03 \); SE: 0.69 and 0.69; \( P = 0.002 \) and < 0.001, respectively, without and with adjustment). Spadano et al (42) also recently concluded that PAL is not independent of body weight in children. This negative confounding could possibly explain why some studies found no differences in PAL between lean and obese children (5, 10, 11). It is well established that obese children expend more energy in a given activity than do nonobese children (9, 43). Consequently, when body size in energy-derived activity measures is not properly adjusted for, the higher energy cost of weight-bearing activities in heavier children could mask real differences in activity levels between lean and overweight children.

With the use of accelerometry, Reilly et al (37) reported recently that young children spend a considerable time in sedentary activities. The number of hours of television viewing, as a marker of sedentary behavior, has been the most consistent risk factor associated with childhood obesity (45, 46). We showed that high levels of light-intensity activity are associated with higher fat tissue mass in boys, but no associations with body composition were found in girls. This finding could be due to the fact that a smaller number of girls completed HR monitoring or could reflect a real sex difference in the effect of light-intensity activity.
on fat accretion. A study of 28 boys aged 9 y also found an association between the time spent in sedentary activity measured by the HR flex method and the %BF measured by skinfold thicknesses ($r = 0.46$, $P < 0.05$) (20). However, the investigators did not find an association between AEE and body fat.

We observed that children aged 6–8 y preferred to spend most of their time in low-to-moderate activity, particularly on weekends, and this finding is supported by other studies (47). Data from 4-d accelerometry showed that boys and girls aged 4–6 y spent a mean of only 32 and 25 min, respectively, in vigorous activity per day (21). Despite this modest time in vigorous activity, a positive association with %BF was observed in some studies (16, 21, 22). Conversely, we found no associations between time spent in vigorous activity and measures of body fat, which indicates that, in this age group, an undue emphasis may be placed on vigorous activity in relation to risk of obesity. Although young children may be active, their activities tend to be of low intensity and typically are not sustained over extended periods. In adults, high PALs can be achieved if there is sufficient input of moderate activity (48). Perhaps it is not surprising, therefore, that we found no relation between vigorous activity and either AEE or PAL. In the current study, children spent a median of 55% and 37% of their waking hours in activities of moderate and light intensity, respectively. After adjustment was made for FFM, no relation was observed between time spent in light-intensity activities and PAL or AEE. There are several reasons why AEE might not correlate significantly with the time spent in light-intensity activity: such an association might be confounded by variations in the proportion of time spent in other categories of activity or by differences in the energetic efficiency with which specific activities are carried out.

This study shows the critical importance of adjustment for body composition and normalization for body size in energy-derived activity measures when assessing relations between measures of PA and body fatness. Although no significant differences in activity were apparent between prepubescent children at various risks of future obesity, even at this young age, differences in activity were apparent between prepubescent children and adults (21). Despite this modest time in vigorous activity, a positive association with %BF was observed in some studies (16, 21, 22). Conversely, we found no associations between time spent in vigorous activity and measures of body fat, which indicates that, in this age group, an undue emphasis may be placed on vigorous activity in relation to risk of obesity. Although young children may be active, their activities tend to be of low intensity and typically are not sustained over extended periods. In adults, high PALs can be achieved if there is sufficient input of moderate activity (48). Perhaps it is not surprising, therefore, that we found no relation between vigorous activity and either AEE or PAL. In the current study, children spent a median of 55% and 37% of their waking hours in activities of moderate and light intensity, respectively. After adjustment was made for FFM, no relation was observed between time spent in light-intensity activities and PAL or AEE. There are several reasons why AEE might not correlate significantly with the time spent in light-intensity activity: such an association might be confounded by variations in the proportion of time spent in other categories of activity or by differences in the energetic efficiency with which specific activities are carried out.

We thank Antony Wright for the stable isotope analysis. MBEL, AMP, and SAJ were responsible for the study design; MBEL and AM were responsible for the acquisition of the data; and WAC was responsible for the statistical analysis. KLR, JCKW, and MBEL were responsible for the interpretation of the data and drafting the manuscript. All authors contributed to the revision of the manuscript. KLR is the guarantor of the study. None of the authors had any personal or financial conflicts of interest.

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Resting metabolic rate is an important predictor of serum adiponectin concentrations: potential implications for obesity-related disorders\textsuperscript{1–3}

\textbf{Johannes B Ruige, Dominique P Ballaux, Tohru Funahashi, Ilse L Mertens, Yuji Matsuzawa, and Luc F Van Gaal}

\section*{ABSTRACT}
\textbf{Background:} Little is known about the regulation of adiponectin. Animal studies suggest local regulation by adipocytokines or alterations in energy expenditure, and studies in humans suggest regulation by alcohol intake and ethnicity.

\textbf{Objective:} To identify regulators of adiponectin in humans, we measured resting metabolic rate (RMR), serum adiponectin, glucose, insulin, triacylglycerol, alcohol intake, and anthropometric indexes in 457 white patients with overweight or obesity.

\textbf{Design:} A cross-sectional design was used, and multivariate regression analysis was performed with adiponectin as the dependent variable and potential predictors as independent variables.

\textbf{Results:} Simple linear analyses showed significant associations between adiponectin and sex, with a standardized coefficient of −0.38 (women compared with men) and an explanation of variation of the model ($R^2$) of 14%; age (0.21; 4%); RMR (−0.52; 27%); fat-free mass (−0.40; 16%); fat mass (−0.16; 2%); visceral fat (−0.24; 6%); computed tomography at L4–L5; fasting triacylglycerol (−0.28; 8%); and insulin resistance (−0.38; 14%); homeostasis model assessment). Adiponectin and alcohol were not associated (−0.04; 0%). Multivariate analyses, which allowed adjustment for confounding, showed that RMR is the most important predictor of adiponectin (−0.31; 29%), followed successively by insulin resistance (−0.16; 31%; model containing RMR and insulin resistance), fat mass (0.20; 34%), age (0.34; 35%), visceral fat (−0.34; 40%), and fasting triacylglycerol (−0.12, 41%).

\textbf{Conclusions:} Low resting metabolism (RMR) is associated with high serum adiponectin. We speculate that subjects with low RMR, who are theoretically at greater risk of obesity-related disorders, are especially protected by adiponectin. \textit{Am J Clin Nutr} 2005;82:21–5.

\textbf{KEY WORDS} Basal metabolism, adiponectin, obesity, metabolic syndrome X, insulin resistance, body constitution

\section*{INTRODUCTION}
Recent progress in obesity research has shown that adipocytes are not merely fat-storing cells but that they secrete a variety of hormones, cytokines, growth factors, and other bioactive substances, conceptualized as adipocytokines. A disturbance of regulation of these adipocytokines contributes to the pathogenesis of obesity-related disorders such as insulin resistance, type 2 diabetes, dyslipidemia, endothelial dysfunction, and vascular disease (1). In particular, the adipocytokine adiponectin has been shown to play an important role; it exerts insulin-sensitizing and antiatherogenic effects (2). The adiponectin knockout (KO) mice, for example, exhibited severe diet-induced insulin resistance with reduced insulin receptor substrate–1–associated phosphatidylinositol 3 kinase activity in muscle. The KO mice also showed neointimal thickening in response to vascular injury and hypertension induced by salt diet. These phenotypes in KO mice were reversed by viral-mediated production of adiponectin (3). A causal relation between decreased plasma adiponectin and insulin resistance and atherosclerosis has been suggested in humans as well (4, 5).

Unfortunately, we do not sufficiently know how adiponectin concentration is regulated. Local effects of tumor necrosis factor-\textalpha, interleukin-6, \beta-adrenoceptor agonist, glucocorticoids (6), specific receptors on the adipocyte, environmental effects such as alcohol intake, and genetic effects, eg, ethnicity, have been suggested (7, 8). In addition, energy expenditure might affect adiponectin, as suggested by animal models (9). In general, energy expenditure is required to maintain basic physiologic functions (eg, heart beat, muscle contraction, respiration); to metabolize, digest, and store consumed food; and to perform physical activity (10). A relation between physical activity and adiponectin could not be established by some recent investigators (11, 12).

To explore the role of energy expenditure in the regulation of adiponectin, we measured basal metabolism or resting metabolic rate (RMR), the energy expenditure required to maintain basic physiologic functions, as well as other potential regulators or confounders and explored their relation with serum adiponectin in overweight or obese patients.

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\textsuperscript{5} Accepted for publication March 9, 2005.
SUBJECTS AND METHODS

Patients with overweight or obesity were recruited from the outpatient clinic of the Department of Diabetology, Metabolism, and Clinical Nutrition of the University Hospital, Antwerp, Belgium. Patients with endocrine disorders (eg, hypothalamic-pituitary disorders, hypothyroidism, Cushing’s syndrome), genetic (dysmorphic) disorders, obesity, or diabetes mellitus type 2 defined according to World Health Organization criteria (13) were excluded. The study was performed according to the standards on human experimentation in accordance with the Helsinki Declaration of 1975 as revised in 1983. Smoking and alcohol habits were estimated by careful questioning, and individuals were divided into dichotomous categories. Individuals who had quit smoking were defined as nonsmokers, and individuals who did not use alcohol on a daily basis were defined as nonalcohol drinkers.

Anthropometry and resting metabolic rate

Height was measured to the nearest 0.5 cm, body weight was measured with a digital scale to the nearest 0.1 kg, body mass index (in kg/m²) was calculated, and percentage of body fat and fat-free mass (FFM; in kg) were assessed by bioimpedance (SEAC SFB3; SEAC, Brisbane, Australia) as described by Lukaski et al (14). Visceral and subcutaneous fat were assessed by a computerized tomography scan at the L4–L5 level, determined according to the technique described by Van der Kooy and Seidell (15) and Kvist et al (16). The RMR is the amount of energy expended when an adult organism is awake but resting, fasting, and at thermal neutrality. RMR was measured and the respiratory quotient was calculated as \( \frac{V\dot{CO}_2}{V\dot{O}_2} \), as reported previously (17). In short, RMR was measured by indirect calorimetry with a ventilated hood system (Deltatrac; Datex, Helsinki, Finland). Subjects stayed overnight at the metabolic ward of the University Hospital Antwerp, and RMR was measured in the morning on awakening after an overnight fast. Oxygen consumption and carbon dioxide production in expired air were measured each minute for 30 min after a 10-min equilibration period. Energy expenditure was calculated with the equation of de Weir (18). In addition to energy expenditure, substrate oxidation was calculated with the equations of Lusk (19).

Laboratory analyses

A fasting blood sample was drawn for measurements of glucose, insulin, triacylglycerol, and adiponectin. Plasma glucose was measured with the glucose oxidase method (on Vitros 750 XRC; Ortho Clinical Diagnostics Inc, Rochester, NY). Fasting triacylglycerol was measured on Vitros 750XRC (Ortho Clinical Diagnostics, Johnson & Johnson, Raritan, NJ). Insulin was measured by a radioimmunoassay with the use of Pharmacia Insulin RIA (Pharmacia Diagnostics, Uppsala, Sweden). This assay shows 41% cross-reactivity with proinsulin. Insulin resistance was calculated by the homeostasis model assessment (HOMA) method with the use of fasting plasma glucose and insulin concentrations. Assuming that normal-weight subjects <35 y have an insulin resistance of 1, the value for insulin resistance can be assessed by the following equation:

\[
\text{Fasting insulin (µU/mL)} \times \text{fasting glucose (mmol/L)}/22.5 \ (I)
\]

Results from the HOMA method correlate well with measurements obtained by means of the euglycemic clamp technique. The CV is reported to be between 7.8% and 11.7% (20, 21). Plasma adiponectin concentration was measured by enzyme-linked immunosorbent assay (Otsuka Pharmaceutical Co, Tokushima, Japan) as described previously (22).

Statistical analysis

Study population characteristics and anthropometric and laboratory measurements were presented as proportions or median values with their range and the 25th and 75th percentile. To show the associations between adiponectin and anthropometric measurements, after control for confounding influence of sex and age, partial Pearson’s correlation coefficients were presented. A \( P \) value < 0.05 was regarded as statistically significant. To explore the relation between adiponectin and potential variables,

### TABLE 1

Study population characteristics and anthropometric and laboratory measurements

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>Minimum–maximum</th>
<th>25th–75th percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>41</td>
<td>18–75</td>
<td>32–50</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>106</td>
<td>60–166</td>
<td>95–119</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.68</td>
<td>1.41–1.97</td>
<td>1.62–1.74</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>38</td>
<td>22–60</td>
<td>34–42</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>51</td>
<td>35–92</td>
<td>46–60</td>
</tr>
<tr>
<td>Total fat mass (kg)</td>
<td>53</td>
<td>20–92</td>
<td>44–62</td>
</tr>
<tr>
<td>RMR (kcal/24 h)</td>
<td>1875</td>
<td>1030–3120</td>
<td>1672–2127</td>
</tr>
<tr>
<td>RMR/FFM (kcal · kg⁻¹ · 24 h⁻¹)</td>
<td>35</td>
<td>26–47</td>
<td>33–38</td>
</tr>
<tr>
<td>Respiratory quotient</td>
<td>0.76</td>
<td>0.70–1.00</td>
<td>0.74–0.79</td>
</tr>
<tr>
<td>Subcutaneous fat (cm²)</td>
<td>605</td>
<td>218–1026</td>
<td>504–709</td>
</tr>
<tr>
<td>Visceral fat (cm²)</td>
<td>156</td>
<td>34–554</td>
<td>111–209</td>
</tr>
<tr>
<td>Fasting serum glucose (mg/dL)</td>
<td>82</td>
<td>63–110</td>
<td>77–89</td>
</tr>
<tr>
<td>Fasting serum insulin (µU/mL)</td>
<td>17</td>
<td>0.2–82</td>
<td>12–24</td>
</tr>
<tr>
<td>Calculated insulin resistance (HOMA)</td>
<td>3.5</td>
<td>0.04–18.2</td>
<td>2.3–5.2</td>
</tr>
<tr>
<td>Fasting serum triacylglycerol (mg/dL)</td>
<td>138</td>
<td>46–568</td>
<td>103–185</td>
</tr>
<tr>
<td>Fasting serum adiponectin (µg/mL)</td>
<td>8.3</td>
<td>1.5–31.8</td>
<td>5.8–11.5</td>
</tr>
</tbody>
</table>

\( n = 457 \). Women composed 75% of the study population. FFM, fat-free mass; RMR, resting metabolic rate; HOMA, homeostasis model assessment.
linear regression analysis was applied. Linear regression analysis provides insight into linear associations and allows adjustment for the influence of potential confounders. If appropriate, variables were normalized by transformation into their natural logarithm to improve the plots of residual analyses. Results were expressed as standardized coefficients and $R^2$, or proportion of variation “explained” by the predictor of interest. Analyses were performed with the SPSS-PC software, version 11.0.1 (SPSS Inc, Chicago, IL).

RESULTS

Four hundred fifty-seven patients were recruited with a median body mass index of 38 (range: 22-60), and 75% were women. The population characteristics and anthropometric and laboratory measurements are shown in Table 1.

Men as well as women with a low RMR expressed per kilogram FFM have higher adiponectin concentrations (Table 2). This association between adiponectin and RMR is further explored in Table 3, which confirms a negative correlation between adiponectin and RMR ($-0.36, P < 0.001$), after control for confounding influence of age and sex. Also shown in Table 3 are significant inverse correlations of adiponectin to insulin resistance, triacylglycerol, and various anthropometric measurements, of which the strongest is visceral fat ($-0.38, P < 0.001$). The RMR is also strongly correlated to insulin resistance, triacylglycerol, and various anthropometric indicators, of which the strongest is FFM (0.73, $P < 0.001$), as has clearly been established previously (10, 23).

To further explore the regulation of serum adiponectin and to allow control for confounding, linear regression models were built with adiponectin as the dependent variable and potential predictors and confounders as independent variables, as shown in Table 4. In the simple linear analyses, various variables were associated with adiponectin: sex, age, RMR, FFM, fat mass, visceral fat, fasting triacylglycerol, and insulin resistance. Adiponectin was not associated with alcohol in the present study, which might be the result of a limitation in precision of assessment of alcohol intake.

On the basis of the results of Table 4, one multivariate regression model was built using the stepwise regression procedure. The strongest correlate appeared to be the RMR, which explained 29% of the variation of adiponectin (Table 5). The second strongest correlate appeared to be insulin resistance, which explained, together with the RMR, 31% of the variation of adiponectin, followed successively by fat mass (34%), age (35%), visceral fat (40%), and fasting triacylglycerol (41%). These results clearly show that adiponectin and RMR are strongly and inversely associated. Additional adjustment for potential confounders, such as age, visceral fat, or HDL cholesterol (data not shown), did not affect the relation between adiponectin and RMR significantly. Using the same variables in a backward procedure resulted in similar findings (data not shown). The results of these multivariate analyses also show the importance of fasting triacylglycerol concentrations in regulation of adiponectin. The model that included RMR, insulin resistance, fat mass, age, visceral fat, and fasting triacylglycerol explained 41% of the variation of adiponectin (Table 5). When HDL cholesterol was included in the model, fasting triacylglycerol would be excluded by the stepwise

---

**Table 2**

Serum adiponectin concentrations according to tertiles of the resting metabolic rate (RMR) per fat-free mass (FFM)

<table>
<thead>
<tr>
<th>Tertile of RMR/FFM (kcal·kg$^{-1}$·24 h$^{-1}$)</th>
<th>1st ($&lt; 34$)</th>
<th>2nd</th>
<th>3rd ($&gt; 37$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum adiponectin ($\mu$g/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Women ($n = 175$)</td>
<td>10.7</td>
<td>10.2</td>
<td>8.2</td>
</tr>
<tr>
<td>Men ($n = 52$)</td>
<td>7.7</td>
<td>5.8</td>
<td>5.0</td>
</tr>
</tbody>
</table>

$^\dagger$ The interaction of sex and tertile was not significant, $P = 0.73$. The main effects of sex ($P = 0.008$) and of tertile of RMR/FFM ($P = 0.001$) were significant.

---

**Table 3**

Partial Pearson’s correlation coefficients between adiponectin and various potential predictors, adjusted for sex and age

<table>
<thead>
<tr>
<th>Adiponectin</th>
<th>RMR</th>
<th>Total fat mass</th>
<th>FFM</th>
<th>BMI</th>
<th>Visceral fat</th>
<th>Subcutaneous fat</th>
<th>Insulin resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMR</td>
<td>$-0.36^2$</td>
<td>$-0.15^2$</td>
<td>$-0.23^2$</td>
<td>$-0.23^3$</td>
<td>$-0.38^2$</td>
<td>$-0.28^2$</td>
<td>$-0.26^2$</td>
</tr>
<tr>
<td>Total fat mass</td>
<td>$-0.65^2$</td>
<td>$0.65^2$</td>
<td>$0.73^2$</td>
<td>$0.67^2$</td>
<td>$0.50^2$</td>
<td>$0.43^2$</td>
<td>$0.28^2$</td>
</tr>
<tr>
<td>FFM</td>
<td>$0.47^2$</td>
<td>$0.88^2$</td>
<td>$0.79^2$</td>
<td>$0.49^2$</td>
<td>$0.41^2$</td>
<td>$0.30^2$</td>
<td>$0.28^2$</td>
</tr>
<tr>
<td>BMI</td>
<td>$0.57^2$</td>
<td>$0.44^2$</td>
<td>$0.73^2$</td>
<td>$0.55^2$</td>
<td>$0.27^2$</td>
<td>$0.23^2$</td>
<td>$0.16^2$</td>
</tr>
<tr>
<td>Visceral fat</td>
<td>$0.55^2$</td>
<td>$0.37^2$</td>
<td>$0.32^2$</td>
<td>$0.32^2$</td>
<td>$0.23^2$</td>
<td>$0.01$</td>
<td>$0.11$</td>
</tr>
<tr>
<td>Subcutaneous fat</td>
<td>$0.09$</td>
<td>$0.12^2$</td>
<td>$0.01$</td>
<td>$0.11$</td>
<td>$0.00$</td>
<td>$0.00$</td>
<td>$0.00$</td>
</tr>
<tr>
<td>Insulin resistance</td>
<td>$0.09$</td>
<td>$0.12^2$</td>
<td>$0.01$</td>
<td>$0.11$</td>
<td>$0.00$</td>
<td>$0.00$</td>
<td>$0.00$</td>
</tr>
</tbody>
</table>

$^1$ RMR, resting metabolic rate; FFM, fat-free mass.

---

**Table 4**

Results from simple linear regression analyses with serum adiponectin as dependent variable and various potential predictors as independent variables

<table>
<thead>
<tr>
<th>Potential predictors in the model</th>
<th>$\beta$</th>
<th>SE</th>
<th>$P$</th>
<th>$R^2$ of model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (female vs male)</td>
<td>$-0.38$</td>
<td>0.05</td>
<td>0.001</td>
<td>0.14</td>
</tr>
<tr>
<td>Age (y)</td>
<td>0.21</td>
<td>0.002</td>
<td>0.001</td>
<td>0.04</td>
</tr>
<tr>
<td>Resting metabolic rate (kcal/24 h)</td>
<td>$-0.52$</td>
<td>0.000</td>
<td>0.001</td>
<td>0.27</td>
</tr>
<tr>
<td>Fat-free mass (kg)</td>
<td>$-0.40$</td>
<td>0.002</td>
<td>0.001</td>
<td>0.16</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>$-0.16$</td>
<td>0.002</td>
<td>0.001</td>
<td>0.02</td>
</tr>
<tr>
<td>Visceral fat (cm$^2$)</td>
<td>$-0.24$</td>
<td>0.000</td>
<td>0.001</td>
<td>0.06</td>
</tr>
<tr>
<td>Fasting triacylglycerol (mg/dL)</td>
<td>$-0.28$</td>
<td>0.000</td>
<td>0.001</td>
<td>0.08</td>
</tr>
<tr>
<td>Insulin resistance (HOMA$^f$)</td>
<td>$-0.38$</td>
<td>0.009</td>
<td>0.001</td>
<td>0.14</td>
</tr>
<tr>
<td>Alcohol (no vs yes)</td>
<td>$-0.04$</td>
<td>0.048</td>
<td>0.933</td>
<td>0.00</td>
</tr>
</tbody>
</table>

$^f$ HOMA, homeostasis model assessment.
procedure. Because triacylglycerol and HDL cholesterol are inversely and undeniably associated and because increased triacylglycerol concentrations more clearly explain their contribution to the development of the metabolic syndrome (24), fasting triacylglycerol instead of HDL cholesterol was included in the final model.

DISCUSSION

We report here on a significant link between serum adiponectin concentrations and RMR. The present study shows high serum adiponectin concentrations in subjects with low RMR. We speculate that protection by adiponectin against obesity-related disorders is especially important for subjects with low RMR. Theoretically, subjects with low RMR are at increased risk of developing these disorders; a larger portion of their daily food intake is stored as fat, in the situation of similar calorie intake. However, the literature reports discrepant findings between RMR and the risk of developing obesity (10, 23, 25). We speculate that this discrepancy in the literature can be explained by a difference in magnitude of protection mechanisms; high risk can be tempered by a better protection by adiponectin.

A disadvantage of the present cross-sectional study is that it does not allow definite conclusions with respect to cause and effect. RMR might affect adiponectin or vice versa. Previous animal studies showed an increase in adiponectin after exposure to cold and suggest a link, not only between adiponectin and thermogenesis but also between adiponectin and genetic-, instead of nutrition-induced obesity (9). Other rodent studies suggest regulation of energy expenditure by adiponectin (26, 27). The latter might be the case as well, but analyses in the present study were performed to test the hypothesis that adiponectin is regulated by energy expenditure. It is, however, also possible that a third underlying unknown factor, eg, genetics, affects both energy expenditure and adiponectin.

In the present study, neither physical activity nor thermogenesis was measured. A link between adiponectin and physical activity is unlikely (11, 12). Except for the previously mentioned report on adiponectin and thermogenesis (9), which represents a maximum 10% of the RMR, to the best of our knowledge, our present study is the first to correlate RMR and adiponectin concentrations.

The present study confirms the earlier described link between adiponectin and triacylglycerol concentrations (28, 29). Increased concentrations of free fatty acid (FFA) and triacylglycerol may ultimately cause an abnormal triacylglycerol storage, which results, in turn, in an increased FFA flux from adipose tissue to nonadipose tissue, which participates in and amplifies many of the fundamental derangements of the metabolic syndrome (24). A recent report mentioned a decrease of adiponectin concentrations after lowering of FFA (30) and suggested an influence of adiponectin on FFA and triacylglycerol (31); thus, a feedback mechanism may exist.

The present study also shows that the inverse association between adiponectin and visceral fat increases after adjustment for fat mass (Table 5), which implies that adiponectin, exclusively secreted by the fat cell, is especially compromised in subjects with visceral obesity (32). This finding, together with the well-known inverse relation between adiponectin and insulin resistance, fit in the same framework and confirm earlier findings on the important interplay between adiponectin and the pathogenesis of the metabolic syndrome (33).

In conclusion, an inverse association between basal metabolism and serum adiponectin concentrations was shown. This inverse association might point to protection by adiponectin against obesity-related disorders particularly when low RMR is present, which itself is theoretically associated with development of obesity (and related disorders).

We thank Sachio Tanaka for technical assistance and the nurses of the outpatient clinic of the Department of Diabetology, Metabolism, and Clinical Nutrition at the University Hospital of Antwerp for their assistance.

LFG was the main investigator, who coordinated and monitored the study. JBR performed the statistical analyses. All authors participated in evaluating the results and in the writing and editing of the manuscript. None of the authors had any financial conflicts related to the work.

REFERENCES

Folic acid supplementation for 3 wk reduces pulse pressure and large artery stiffness independent of MTHFR genotype\(^1\sim3\)

Carolyn Williams, Bronwyn A Kingwell, Kevin Burke, Jane McPherson, and Anthony M Dart

**ABSTRACT**

**Background:** Folic acid reduces plasma homocysteine and may be an important therapy for preventing cardiovascular disease. A key mechanism may be the reduction of arterial stiffness.

**Objective:** The effect of folic acid supplementation on blood pressure and large artery stiffness was examined in relation to methyltetrahydrofolate reductase (MTHFR) genotype.

**Design:** Forty-one asymptomatic men with normal or high-normal ambulatory blood pressure (systolic: >130 to ≤145 mm Hg; diastolic: >80 to ≤90 mm Hg) participated. The study had a randomized, placebo-controlled, double-blind, crossover design that incorporated 3-wk treatments with 5 mg folic acid/d or matching placebo; each treatment was separated by a 4-wk washout phase.

**Results:** Folic acid reduced brachial pulse pressure by 4.7 ± 1.6 mm Hg (P < 0.05) without changing mean arterial pressure. Systemic arterial compliance increased by 0.15 ± 0.03 mL/mm Hg (P < 0.05) after folic acid treatment but did not change after placebo treatment. These responses did not significantly correlate with either homocysteine or folate plasma concentrations. MTHFR genotype CC homozygotes (without the 677C→T polymorphism) with normal blood pressure had a larger reduction in homocysteine concentrations in response to folic acid than did \(T\) allele carriers. Blood pressure and arterial stiffness responses were independent of MTHFR genotype.

**Conclusion:** Folic acid is a safe and effective supplement that targets large artery stiffness and may prevent isolated systolic hypertension. *Am J Clin Nutr* 2005;82:26–31.

**KEY WORDS** Homocysteine, genetics, MTHFR gene, folate therapy, arterial stiffness, pulse pressure, hypertension

**INTRODUCTION**

Hyperhomocysteinemia is emerging as an independent predictor of stroke (1), myocardial infarction (2), atherosclerosis (3, 4), systolic hypertension (5), and cardiovascular disease death (6). In a recent meta-analysis, a 5 mmol/L increase in serum homocysteine was associated with an increase in the risk of stroke and ischemic heart disease by >50% and 30%, respectively (7). The mechanisms underlying the relation between elevated homocysteine concentrations and cardiovascular disease risk may partly relate to increased large artery stiffness and impaired endothelium-dependent vasodilation (8–10).

Folate status is one of the most important determinants of plasma homocysteine concentrations (11, 12), and folic acid supplementation significantly and safely improves endothelial dysfunction in patients with coronary artery disease (13, 14). The effects of folic acid supplementation on large artery stiffness have not been investigated. However, interventions that reduce plasma homocysteine concentrations also lower pulse pressure, which implicates a reduction in large artery stiffness as the mechanism (15). Furthermore, hyperhomocysteinemia induced by folate restriction promotes arterial stiffening (16). Given that large artery stiffness is an independent risk factor for cardiovascular disease (17–19), the effect of folic acid supplementation on the relation between folate status, homocysteine concentrations, and large artery stiffness is of great clinical relevance.

In addition to the role of dietary intake of substrates and vitamin cofactors in homocysteine metabolism, the methylenetetrahydrofolate reductase (MTHFR) gene regulates folate-dependent remethylation of homocysteine to methionine. The 677C→T substitution polymorphism within this gene causes thermolability and reduced activity of the enzyme. It has been suggested that this mutation accelerates the onset of coronary artery disease in patients with familial hypercholesterolemia or a previous myocardial infarction (20, 21).

Folic acid supplements are well tolerated and may improve vascular function both in healthy individuals and in those at elevated risk of cardiovascular disease (15, 22, 23). Our study examined the effect of folic acid supplementation on blood pressure and large artery stiffness in healthy individuals and in patients with early stage systolic hypertension. We elected to study young individuals with normal or mildly elevated blood pressure on the premise that they would be more responsive to short-term folic acid treatment than would older individuals with irreversibly stiffened large arteries (24). In addition, a beneficial effect in these groups may have implications for the prevention of isolated systolic hypertension. All variables were assessed in relation to the MTHFR 677C→T genotype with the hypothesis that the \(T\) allele would be associated with reduced folate responses.

**SUBJECTS AND METHODS**

**Subjects**

Twenty normotensive participants (ambulatory blood pressure ≤130/80 mm Hg) and 21 participants with high-normal...
blood pressure [ambulatory blood pressure: >130 to <145 mm Hg (systolic) and >80 to <90 mm Hg (diastolic)] were recruited. The participants were all disease-free and were categorized by their 24-h ambulatory blood pressure rather than by their “office” blood pressure, which is obtained by taking the average of 3 blood pressure measurements taken 3 min apart after 5–10 min rest in a supine position. We had several reasons for measuring 24-h ambulatory blood pressure: 1) it correlates better with end organ damage, 2) it predicts cardiovascular disease risk better, and 3) it is more reproducible and independent of the “white coat” effect (25). All participants were men, were aged 20–40 yrs, were nonsmokers, were nonusers of medication, were healthy (other than high blood pressure in some), and had no history of cardiovascular disease, diabetes, or liver disease. Two participants were taking medication for high blood pressure, which was discontinued 2 wk before the study began. The study was approved by the Alfred Hospital ethics committee. Each participant gave written informed consent before commencing the study.

Protocol

A randomized, placebo-controlled, double-blind, crossover design was used. The participants were randomly assigned to a 3-wk treatment with either 5 mg folic acid/d or matching placebo. After the first 3-wk phase, the participants entered a washout phase for 4 wk and were then crossed over to the alternate study arm to complete the final 3-wk intervention phase. A daily dose of 5 mg folic acid/d is the conventional clinical dose (26–28) that effectively lowers plasma homocysteine after 3 wk (29) and improves endothelial function after 4 wk (22), with such effects abolished after a 4-wk washout phase (23). The intervention duration of 3 wk was chosen to maximize participant adherence to the protocol. The participants fasted 10–12 h overnight before the 4 study visits and, on arrival, rested in a supine position in a temperature-controlled laboratory. After a period of 5 min, blood pressure was measured followed by measurements of arterial stiffness. Blood samples were collected by venipuncture immediately after the arterial stiffness measurements. Supine brachial blood pressure and heart rate were determined with an automated oscillometric blood pressure monitor (Dinamap Vital Signs Monitor 1846SX; Critikon) and used to calibrate the carotid arterial pressure contour. This method has been validated against invasive pressure recordings (34, 35).

PWV was measured centrally (between the right carotid and femoral arteries) and peripherally (between the right femoral and dorsal pedis arteries) by simultaneous applation tonometry (SPT-301; Millar Instruments) as described previously (36).

Biochemical analysis

Plasma total cholesterol, LDL cholesterol, HDL cholesterol, triacylglycerols, and glucose were measured with a bench-top analyzer (Cholestech LDX; Cholestech Corp, Hayward, CA). Samples for homocysteine analysis were placed immediately on ice, centrifuged at 1500 × g for 10 min at 4 °C, and frozen at −80 °C within 2 h of collection. Plasma homocysteine concentrations were measured with an Abbott AxSYM immunochemistry analyzer assay with fluorescence polarization immunoassay technology (Axis Biochemicals ASA, Oslo, Norway). The normal homocysteine range is 5–15 μmol/L.

MTHFR genotyping

DNA was isolated with the PureGene DNA purification system (Gentra Systems, Plymouth, MN). The 677C→T polymorphism was detected by polymerase chain reaction as described by Froost et al (37).

Statistics

All data are presented for the total population (ie, normal and high-normal blood pressure combined). Baseline data are presented as means ± SDs. Intervention data are presented as means ± SEs of the difference (SED). The effects of folic acid or placebo intervention were compared with repeated-measures analysis of variance, with variable change as the dependent factor and the order of intervention, genotype, and blood pressure group (normal or high-normal) as independent factors. Proportional data were analyzed by Fisher’s exact test. All analyses were done by using SPSS (version 11.5; SPSS Inc, Chicago, IL).

RESULTS

Baseline data are presented in Table 1 and Table 2. All participants completed the study and no adverse effects were
TABLE 1
Baseline characteristics of the patients\(^1\)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value (n = 41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>32 ± 7</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>85 ± 17</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>179 ± 8</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.90 ± 0.03</td>
</tr>
<tr>
<td>Plasma cholesterol (mmol/L)</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td>Plasma triacylglycerol (mmol/L)</td>
<td>1.4 ± 1.0</td>
</tr>
<tr>
<td>Plasma HDL (mmol/L)</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>Plasma LDL (mmol/L)</td>
<td>3.1 ± 0.9</td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>5.2 ± 0.6</td>
</tr>
<tr>
<td>Plasma Hcy (µmol/L)</td>
<td>8.8 ± 1.9</td>
</tr>
<tr>
<td>Total folate intake (mg/d)</td>
<td>316 ± 161</td>
</tr>
<tr>
<td>Plasma folate (nmol/L)</td>
<td>25 ± 7</td>
</tr>
<tr>
<td>RBC folate (nmol/L)</td>
<td>1034 ± 287</td>
</tr>
<tr>
<td>Plasma vitamin B-12 (pmol/L)</td>
<td>281 ± 106</td>
</tr>
<tr>
<td>Plasma creatinine (mol/L)</td>
<td>0.08 ± 0.01</td>
</tr>
</tbody>
</table>

\(^1\) All values are \(\bar{x} \pm SD\). Hcy, homocysteine; RBC, red blood cell.

reported. There were no significant effects due to the order of intervention for any of the reported variables. There was no significant change in plasma creatinine or vitamin B-12 concentrations in response to folic acid treatment (data not shown).

The MTHFR 677C→T genotype frequencies were as follows: 56.0% CC, 31.7% CT, and 12.3% TT; these frequencies did not differ from the Hardy-Weinberg equilibrium. Because of the low frequency of the \(T\) allele, \(CC\) homozygotes were compared with \(T\) allele carriers (\(CT\) and \(TT\)) in subsequent analyses.

Folate and homocysteine concentrations

The 3-wk supplementation with folic acid significantly increased plasma folate and red blood cell folate concentrations and reduced plasma homocysteine concentrations compared with placebo (Figure 1). In those participants with a normal blood pressure, the \(CC\) genotype group had a significantly greater homocysteine response to folic acid supplementation than did the \(T\) allele carriers (\(-1.46 \pm 0.29\) µmol/L for \(CC\) homozygotes compared with \(-0.15 \pm 0.33\) µmol/L for \(T\) allele carriers; \(P < 0.01\)). However, differences between the \(CC\) homozygotes and the \(T\) allele carriers were not significant in those with high-normal blood pressure. Regardless of blood pressure

TABLE 2
Baseline blood pressures and arterial stiffness measurements\(^1\)

<table>
<thead>
<tr>
<th>Measure</th>
<th>Value (n = 41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachial SBP (mm Hg)</td>
<td>131 ± 16</td>
</tr>
<tr>
<td>Brachial DBP (mm Hg)</td>
<td>74 ± 10</td>
</tr>
<tr>
<td>Brachial MAP (mm Hg)</td>
<td>95 ± 12</td>
</tr>
<tr>
<td>Brachial PP (mm Hg)</td>
<td>57 ± 9</td>
</tr>
<tr>
<td>Central PP (mm Hg)</td>
<td>49 ± 12</td>
</tr>
<tr>
<td>SAC (mL/mm Hg)</td>
<td>0.99 ± 0.4</td>
</tr>
<tr>
<td>Aortic PWV</td>
<td>7.2 ± 0.9</td>
</tr>
<tr>
<td>Peripheral PWV</td>
<td>10.6 ± 1.5</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>62 ± 8</td>
</tr>
</tbody>
</table>

\(^1\) All values are \(\bar{x} \pm SD\) of 10 consecutive measurements at baseline. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial blood pressure; PP, pulse pressure; HR, heart rate; SAC, systemic arterial compliance; PWV, pulse wave velocity.

FIGURE 1. Mean (±SED) change in plasma folate, red blood cell (RBC) folate, and plasma homocysteine (Hcy) in the placebo— and folic acid–treated groups. **, ***, ***Significantly different from placebo: *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\).

group, plasma homocysteine concentrations were reduced >1 µmol/L in 59% of \(CC\) homozygotes compared with 22% of \(T\) allele carriers (\(P < 0.05\)). There was no significant difference in the serum or red blood cell folate response to folic acid supplementation between genotype groups.

Blood pressure

Mean and diastolic blood pressures were unaffected by folic acid supplementation (changes in mean pressure were \(-0.8 \pm 1.3\) and \(-1.6 \pm 1.0\) mm Hg for placebo and folate, respectively; respective changes in diastolic pressure were \(-0.3 \pm 1.1\) and \(0.0 \pm 1.0\) mm Hg). Decreases in brachial systolic blood pressure (\(-3.4 \pm 1.0\) compared with \(-0.3 \pm 1.3\) mm Hg) and central systolic blood pressure (\(-1.6 \pm 1.9\) compared with \(-0.3 \pm 2.3\) mm Hg) were greater during folic acid treatment than during placebo treatment, but the differences were not statistically significant. In contrast, both brachial and central pulse pressures were significantly reduced by folic acid compared with placebo intervention (Figure 2). Interactions between the response to folic acid supplementation and genotype or blood pressure group were not statistically significant.

Arterial stiffness

SAC increased significantly in response to folic acid supplementation compared with placebo (Figure 2). This response was unaffected by genotype or blood pressure group.
FOLIC ACID AND ARTERIAL STIFFNESS

Changes in PWV were not significantly different between the folic acid and placebo intervention phases (−0.09 ± 0.21 and 0.19 ± 0.25 m/s peripheral PWV for folic acid and placebo treatments, respectively; −0.10 ± 0.11 and 0.09 ± 0.10 m/s central PWV for folic acid and placebo treatments, respectively). Interactions between the response to folic acid supplementation and genotype or blood pressure group were not statistically significant.

DISCUSSION

The major finding of this study was that short-term dietary supplementation with folic acid reduced pulse pressure in young men with normal or mildly elevated systolic blood pressure. Pulse pressure decreased in the absence of any change in mean arterial pressure and in conjunction with a decrease in arterial stiffness. The effect of folic acid supplementation on pulse pressure is thus likely to be secondary to a reduction in arterial stiffness. There was no significant relation between changes in plasma homocysteine concentrations and changes in blood pressure or arterial stiffness. Although the decrease in homocysteine concentration in response to folic acid supplementation was greater in CC homozygotes of the MTHFR 677C→T mutation than in T allele carriers, blood pressure and arterial stiffness responses were similar in both genotype groupings. These data indicate that, regardless of MTHFR genotype, folic acid supplementation may be effective in the prevention of elevated pulse pressure, a condition that is secondary to elevated arterial stiffness.

A previous study reported an association between plasma homocysteine concentrations, systolic blood pressure, and indexes of arterial stiffness (5), but the effects of folic acid on arterial stiffness have not been studied. Interestingly, although folic acid supplementation increased SAC, both central and peripheral PWV were unchanged. This disparity most likely relates to the fact that these measures assess different arterial territories. The carotid to femoral PWV omits the most proximal and elastic portion of the aorta. The absence of a folic acid–mediated effect on this variable suggests that the changes in arterial stiffness occurred primarily in the proximal aorta. The distal PWV data indicate that folic acid supplementation does not influence the stiffness of smaller conduit arteries.

Mechanisms

Responses to folic acid were confined to effects on pulse pressures; no effects on mean or diastolic pressures were observed. These findings are consistent with the hypothesis that homocysteine reduces large artery stiffness. The absence of an effect on mean and diastolic pressure makes it less likely that the observed changes in arterial stiffness are themselves secondary to blood pressure changes. The folic acid–induced reduction in arterial stiffness is likely related to multiple homocysteine-dependent and -independent mechanisms. Rapid changes in nitric oxide–mediated endothelial function were observed after supplementation with folic acid but before plasma homocysteine changes were detected (38), which implies a role for plasma homocysteine–independent mechanisms. Such effects may be mediated by the antioxidant properties of folic acid (8, 39) and are likely to mediate a rapid reduction in arterial stiffness through a reduction in the catabolism of nitric oxide and an enhancement of endothelial-dependent vasodilation (40). Nestel et al (41) reported rapid increases in arterial stiffness within 2.5 h of a methionine load before homocysteine concentrations changed. Another study suggests that methionine loading may mediate such detrimental effects on arterial stiffness through an impairment of the nitric oxide system (42). Changes in plasma homocysteine concentrations may also influence endothelial-mediated effects on arterial stiffness. Scholze et al (9) reported improvements in endothelial function and a reduction in pulse pressure in patients with end-stage renal failure after intravenous acetylcysteine reduced homocysteine concentrations during hemodialysis. Such effects may relate to a reduction in oxidative stress and in inflammatory factors that are associated with elevated plasma homocysteine (9).

Although effects on endothelial function may rapidly modulate arterial stiffness, inhibition of the destructive effects of homocysteine on the extracellular matrix may mediate long-term structural benefits. Homocysteine increases the expression of elastolytic matrix metalloproteinases, including MMP-2 and MMP-9 (43, 44). This mechanism may explain the elastinolytic erosion of the arterial wall in minipigs after methionine-induced hyperhomocysteinemia (10). Patients with hyperhomocysteinemia released more MMP-9 from peripheral blood mononuclear cells in response to oxidized LDL cholesterol stimulation than did healthy control subjects (45). Folic acid supplementation may thus inhibit the effects of homocysteine-induced extracellular matrix elastolysis and thereby reduce arterial stiffness.
**MTHFR genotype**

Consistent with a previous study (46), T allele carriers of the 677C→T polymorphism had a smaller reduction in homocysteine concentrations in response to folic acid supplementation than did CC homozygotes. Other investigators suggest that T allele carriers have lower baseline plasma folate concentrations, have higher baseline plasma homocysteine concentrations, and are more sensitive to the homocysteine-lowering effect of folic acid supplementation than are CC homozygotes (47–49). In the present study, there was no difference in baseline plasma homocysteine or plasma folate concentrations between genotype groupings. Given this fact and the lower MTHFR enzyme activity in T allele carriers, the smaller homocysteine response in this group was expected. Despite this difference, arterial compliance increased in response to folic acid supplementation independent of genotype. These data are consistent with the hypothesis that folic acid mediates its arterial and hemodynamic effects partly independent of MTHFR activity.

**Clinical relevance**

Both arterial stiffness and pulse pressure have been positively related to cardiovascular and, in particular, coronary outcome (17–19, 50). The underlying mechanism likely relates to the mismatch in cardiac blood supply and demand when the heart ejects into a stiff circulation. This effect is mediated by increased cardiac afterload, secondary to an elevation in systolic pressure and a reduction in coronary perfusion as a consequence of lower diastolic pressure (51–53). In patients with coronary artery disease who have stiffer aortas, this translates to a reduction in myocardial ischemic threshold (54). Interventions that reduce arterial stiffness may thus have efficacy not only in reducing systolic and pulse pressure but also in raising the ischemic threshold. Currently available drugs do not specifically target the large arteries. Our data suggest that folic acid supplementation represents a simple and safe strategy to reduce or even prevent age-related arterial stiffening and pulse pressure elevation. Given that approximately 20% of middle-aged men and women in Australia and 22% in the United States consume less than the recommended daily intake of folate, supplementation with folic acid or fortification of food with B vitamins could have beneficial effects (28, 55). The results from the 5-d dietary intake analysis indicate that the average daily intake of folate in our subject group was above that recommended by the National Health and Medical Research Council of Australia; however, 21% of participants had an intake of folate that was less than the recommended intake. These data imply that the benefits of folic acid supplementation may not be limited to persons with an inadequate dietary intake of folate.

In summary, short-term folic acid treatment reduces pulse pressure and arterial stiffness in young men. This effect was independent of MTHFR genotype. Our data indicate that folic acid is a safe and effective supplement that targets large artery stiffness and may reduce isolated systolic hypertension.

KB and JM provided expert technical assistance during this project. CW, BAK, and AMD were involved in the study conception, design, and analysis and in the writing of the manuscript. None of the authors had any conflicts of interest.

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Functional foods for coronary heart disease risk reduction: a meta-analysis using a multivariate approach¹⁻³

Inar A Castro, Lúcia P Barroso, and Patricia Sinnecker

ABSTRACT

Background: It has been difficult to identify the appropriate bioactive substance for the development of new functional foods associated with coronary heart disease, because the results of many clinical studies are contradictory.

Objective: The objective of this study was to use the multivariate statistical approach known as principal component analysis (PCA) followed by a mixed model to process data obtained from a meta-analysis aimed at evaluating simultaneously the effect of ingestion of 1 of 3 types of bioactive substances (n⁻³ fatty acids, soluble fibers, and phytosterols) on 1 or more of 4 biomarkers (plasma total cholesterol, triacylglycerol, LDL cholesterol, and HDL cholesterol).

Design: Five independent variables (number of patients per study, dose, age, body mass index, and treatment length) and 4 dependent variables (percentage change in blood total cholesterol, LDL, HDL, and triacylglycerol) from 159 studies and substudies were organized into a matrix. The original values were converted to linear correlation units, which resulted in a new matrix.

Results: Two principal components were enough to explain 63.73% and 84.27% of the variance in the independent and dependent variables, respectively. Phytosterols and soluble fibers had a hypocholesterolemic effect, whereas n⁻³ fatty acids lowered triacylglycerol and increased total, LDL, and HDL cholesterol. The PCA and mixed model showed that this behavior was independent of dose, number of patients per study, age, and body mass index but was associated with treatment length.


KEY WORDS

n⁻³ Fatty acids, phytosterols, soluble fibers, cholesterol, multivariate analysis, functional foods

INTRODUCTION

The role of plasma lipids in the etiology of atherosclerosis and coronary heart disease has been well defined. A high plasma concentration of total cholesterol, triacylglycerol, and LDL cholesterol and a low plasma concentration of HDL cholesterol are considered important risk factors for the expression of coronary disease (1), and these plasma indexes or biomarkers must be jointly considered in the assessment of risk for populations (2).

Several studies have shown that the ingestion of bioactive substances—such as certain types of n⁻³ fatty acids (linoleic, eicosapentaenoic, and docosahexaenoic acids), soluble fibers (guar gum, psyllium, pectin, and oat products), and phytosterols (stanols and sterols)—may have a positive and significant lipemic effect (3–5). However, reported contradictory results impair the choice of one or more substances for the development of new foods that could promote a reduction in the risk of coronary heart disease in humans (6–11). For the development of such foods, known as functional foods (12–14), in addition to satisfying all criteria necessary for the formulation of a regular food, one must also assess their functional efficiency on the basis of alterations in biomarkers.

Statistical techniques normally adopted in a meta-analysis, such as general regression models, summarize important information; however, they deal with one variable at a time. Multivariate approaches are statistical procedures capable of promoting data reduction or structural simplification, sorting and grouping, investigating the dependence among variables, predicting, and hypothesis testing. These approaches have been widely used in several areas of research such as medicine, sociology, business, education, psychology, and sports (15). In addition to being very efficient tools, especially in the study of correlations involving a large number of variables and sample units, these approaches have rarely been applied in nutritional research, despite this being an area in which multivariate correlation studies are essential.

The objective of this study was to present the multivariate statistical approach known as principal component analysis (PCA) followed by a mixed model to process data obtained from a meta-analysis aimed at evaluating simultaneously the effect of ingestion of 1 of 3 types of bioactive substances (n⁻³ fatty acids, soluble fibers, and phytosterols) on 1 or more of 4 biomarkers (plasma total cholesterol, triacylglycerol, LDL cholesterol, and HDL cholesterol).

SUBJECTS AND METHODS

Study characteristics

Studies of the effects of some n⁻³ fatty acids, phytosterols, and soluble fiber on blood total cholesterol, LDL-cholesterol, HDL-cholesterol, and triacylglycerol.

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HDL-cholesterol, and triacylglycerol concentrations in adults were identified by a computerized literature search of the Internet [Web of Science, Elsevier, and MEDLINE (National Library of Medicine, Bethesda, MD)] of articles primarily published (96%) from January 1990 to April 2003 and by examining their respective cited reference sources. The full texts were obtained from the Library of the Institute of Chemistry of the University of São Paulo (USP), Brazil, and from the Louise M Darling Biomedical Library of the Center for Health Sciences, University of California. Only published trials reported in English were considered. Studies were selected for analysis according to the criteria shown in Table 1. For this meta-analysis, studies involving supplementation with more than one type of functional ingredient and studies that applied more than one dose or intervention time were partitioned to be treated as substudies. The dependence of these specific studies and substudies was considered in the mixed model.

**Specification of the variables**

In the present meta-analysis, 4 dependent variables (percentage change in cholesterol, LDL cholesterol, HDL cholesterol, and triacylglycerol) and 5 independent variables [number of patients per study, dose, age, body mass index (BMI), and treatment length] were obtained from 159 studies and substudies that were described in detail by the respective authors (Table 2) cited in the reference.

The change in biomarkers (Δ%) was calculated according to the following equation:

\[
\Delta \% \text{Biomarker} = \frac{[(T_1 - T_0)/T_0] - [(P_1 - P_0)/P_0]}{(100)}
\]

**Table 2**

References reviewed in this meta-analysis categorized by the bioactive substance applied in the dietary interventions

<table>
<thead>
<tr>
<th>Bioactive substance</th>
<th>Reference</th>
<th>n²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytosterols</td>
<td>16–43</td>
<td>61</td>
</tr>
<tr>
<td>Soluble fibers</td>
<td>4, 7, 44–61</td>
<td>32</td>
</tr>
<tr>
<td>n=3 Fatty acids</td>
<td>10, 62–90</td>
<td>66</td>
</tr>
</tbody>
</table>

1 The reference numbers correspond to the same studies presented in Figures 3 and 4.

2 Corresponds to the total number of studies and substudies in each bioactive substance group.

where \(T_1, T_0, P_1, \) and \(P_0\) were the final and initial blood concentrations of the specific biomarker for the experimental and the placebo groups, respectively. The number of patients per study was obtained from the total number of individuals included in the study or substudy, with \(\approx 50\%\) corresponding to the experimental and \(50\%\) to the placebo groups. Dose was considered as the functional ingredient in its pure form and was reported in grams. Age (y) and BMI (kg/m²) were obtained from the mean for the experimental and placebo groups at baseline. The treatment length variable was computed with respect to the effective period of intervention, excluding both the adaptive and the washout periods in the crossover studies. Studies whose variables could not be included in this classification were not considered for this meta-analysis.

In general, the studies showed closely similar patterns concerning the form of bioactive substance ingestion, sex, experimental design, subject’s baseline healthy conditions (cholesterol \(< 8\) mmol/L and triacylglycerols \(< 3\) mmol/L), diet (energy \(\approx 2500\) kcal, cholesterol \(= 300\) mg, and total fat \(\approx 30\%\) of energy), moderate practice of physical exercise, alcohol consumption, smoking habit, and use of mild drugs or other nutritional supplements, which were normally discontinued a few months before or maintained without changes during the intervention. Similarities in lifestyles allowed the conclusion that alterations observed in the biomarkers resulted basically from the nutritional interventions.

**Statistical analysis**

PCA was the multivariate technique applied to assess the association between the 4 dependent variables and the 5 independent variables and to categorize the studies and substudies according to the plane generated by the main components. PCA is only a descriptive statistical procedure, which does not involve any supposition about variance homogeneity. It is a simple and adequate descriptive technique to handle quantitative variables. The data matrices for independent variables (159 \(\times 5\)) and for dependent variables (159 \(\times 4\)), expressed in different units (g, kg/m², y, d, and %), were prepared by adopting the variables as columns and the studies and substudies as rows. First, statistical standardization was performed to obtain relativized data to which the multivariate technique was applied. The original values were converted into linear correlation units to form a new matrix, which was used as the base for PCA. The grouping
variables were designated as n—3 fatty acids, soluble fiber, and phytosterols. Correlations between the variables of all selected studies and substudies were used, with the variables and “studies and substudies” being grouped as a function of similarities.

With respect to the variables, the number of factors obtained should be determined by the number of eigenvalues >1.0 (91). Eigenvalues correspond to vectors capable of holding part of the variation observed when the original values (4 and 5) are reduced into the principal components. The studies and substudies were plotted graphically on the two-dimensional plane generated by the variables. A mixed model was fitted using 2 principal components of the dependent variables as response. All calculations were performed by using STATISTICA software (version 6; Statsoft Inc, Tulsa, OK), except those in the mixed model, for which SAS software (SAS Institute Inc, Cary, NC) was used.

RESULTS

Study characteristics

The studies reported the effect of 3 different bioactive substances—n—3 fatty acids, phytosterols, and soluble fibers—on the percentage change in cholesterol, LDL cholesterol, HDL cholesterol, and triacylglycerol under different experimental conditions (Table 3). With this initial approach, it was possible to observe that the general dietary interventions promoted average net reductions in cholesterol, LDL cholesterol, and triacylglycerol of 3.57%, 3.88%, and 11.50%, respectively, and a net increase in HDL cholesterol of 1.79%.

TABLE 3
Summary of variables observed in the studies included in the meta-analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median</th>
<th>x ± SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Independent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of patients per study</td>
<td>39.00</td>
<td>84.28 ± 147.32</td>
<td>159</td>
</tr>
<tr>
<td>Dose (g)</td>
<td>2.76</td>
<td>3.77 ± 3.06</td>
<td>159</td>
</tr>
<tr>
<td>Age (y)</td>
<td>48.65</td>
<td>46.86 ± 11.63</td>
<td>151</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.94</td>
<td>25.64 ± 2.14</td>
<td>144</td>
</tr>
<tr>
<td>Treatment length (d)</td>
<td>42.00</td>
<td>61.49 ± 60.21</td>
<td>159</td>
</tr>
<tr>
<td>Dependent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔCholesterol (%)</td>
<td>−3.76</td>
<td>−3.57 ± 5.34</td>
<td>130</td>
</tr>
<tr>
<td>ΔTriacylglycerol (%)</td>
<td>−11.29</td>
<td>−11.50 ± 13.97</td>
<td>125</td>
</tr>
<tr>
<td>ΔHDL cholesterol (%)</td>
<td>1.43</td>
<td>1.79 ± 5.72</td>
<td>111</td>
</tr>
<tr>
<td>ΔLDL cholesterol (%)</td>
<td>−4.50</td>
<td>−3.88 ± 6.87</td>
<td>115</td>
</tr>
</tbody>
</table>

1 Δ, change.

Multivariate analysis

The eigenvalues obtained by PCA for the independent and dependent variables are presented in Tables 4 and 5, respectively. They were arranged in decreasing order, indicating the importance of the respective factor in explaining the variation of the data. On the basis of the decision criteria recommended by Piggot and Sharman (91), 2 factors (eigenvalues > 1.0) were selected and the factor coordinates of the variables, based on correlations for each one, are presented in Table 6. Two principal components were enough to explain 63.73% and 84.27% of the variance in the independent and dependent variables, respectively. The linear correlations between the dependent and independent variables, including the principal components PCD1 and PCD2 (first and second principal components for dependent variables) and PCI1 and PCI2 (first and second principal components for independent variables), are presented in Table 7. The correlations were low. The correlation between treatment length and the percentage change in cholesterol, LDL cholesterol, PCD1, and PCD2 and the correlation between PCI1 and PCI2 were significantly different from zero. The vectors relative to the centered and reduced variables, selected as active in this meta-analysis, were located on the circumference and are graphically represented in Figures 1 and 2.

The distribution of the 159 studies and substudies on the plane generated by the variables is shown in Figures 3 and 4. The contribution of BMI and age to the first principal component and of treatment length and number of patients per study to the second principal component are shown in Figure 1. The dose variable did not present a high correlation with the 2 principal components discussed in this study. Considering the dependent variables, the

TABLE 5
Partitioning of the factors into principal components for the dependent variables: percentage change in cholesterol, LDL cholesterol, HDL cholesterol, and triacylglycerol

<table>
<thead>
<tr>
<th>Component</th>
<th>Eigenvalue</th>
<th>Total variance</th>
<th>Cumulative eigenvalue</th>
<th>Cumulative variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.86</td>
<td>37.13</td>
<td>1.86</td>
<td>37.13</td>
</tr>
<tr>
<td>2</td>
<td>1.33</td>
<td>26.60</td>
<td>3.19</td>
<td>63.73</td>
</tr>
<tr>
<td>3</td>
<td>0.88</td>
<td>17.63</td>
<td>4.07</td>
<td>81.36</td>
</tr>
<tr>
<td>4</td>
<td>0.62</td>
<td>12.41</td>
<td>4.69</td>
<td>93.77</td>
</tr>
<tr>
<td>5</td>
<td>0.31</td>
<td>6.23</td>
<td>5.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

1 Δ, change.

TABLE 6
Factor-variable correlations (factor loadings) based on correlations

<table>
<thead>
<tr>
<th>Variable</th>
<th>Factor loading 1</th>
<th>Factor loading 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Independent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of patients per study</td>
<td>0.42</td>
<td>−0.72</td>
</tr>
<tr>
<td>Dose</td>
<td>0.40</td>
<td>0.43</td>
</tr>
<tr>
<td>Age</td>
<td>0.89</td>
<td>0.11</td>
</tr>
<tr>
<td>BMI</td>
<td>0.79</td>
<td>0.34</td>
</tr>
<tr>
<td>Treatment length</td>
<td>0.33</td>
<td>−0.71</td>
</tr>
<tr>
<td>Dependent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔCholesterol</td>
<td>−0.83</td>
<td>−0.52</td>
</tr>
<tr>
<td>ΔTriacylglycerol</td>
<td>0.56</td>
<td>−0.70</td>
</tr>
<tr>
<td>ΔHDL cholesterol</td>
<td>−0.68</td>
<td>0.45</td>
</tr>
<tr>
<td>ΔLDL cholesterol</td>
<td>−0.92</td>
<td>−0.29</td>
</tr>
</tbody>
</table>

1 Δ, change.
influence of the percentage change in LDL cholesterol, cholesterol, HDL cholesterol, and triacylglycerol on the first principal component and the influence of triacylglycerol on the second principal component are shown in Figure 2.

The mixed-model analysis was performed for the first 2 dependent principal components. The final equations are presented in Table 8. In the initial models, the bioactive substances (n−3 fatty acids, phytosterols and soluble fibers) were included as a fixed effect, studies as a random effect, and PCI1 and PCI2 as well as the interaction between type of study and independent principal components as exploratory variables. The mixed-model analysis showed that the bioactive substances and PCI2 had a statistically significant effect. The interaction effects and the main effect of the first independent principal component were removed from the model because none of them were statistically significant at a 0.05 level of significance. A multiple comparisons Tukey’s test was applied to compare the 3 intercepts corresponding to the type of study. For the first dependent principal component, P values adjusted with the Tukey-Kramer test were as follows: phytosterols versus soluble fibers (P = 0.2310), n−3 fatty acids versus phytosterols (P < 0.0001), and n−3 fatty acids versus soluble fibers (P < 0.0001). This result indicates that the intercept for phytosterols and soluble fibers is equal and both variables are different from the intercept for n−3 fatty acids. For the second dependent principal component, P values adjusted with the Tukey-Kramer test were as follows: n−3 fatty acids versus phytosterols (P = 0.7843), n−3 fatty acids versus soluble fibers (P = 0.1022), and phytosterols versus soluble fibers (P = 0.0154). The conclusion is that the intercept for n−3 fatty acids and phytosterols is equal and both of them are different from the intercept for soluble fibers.

A residual analysis was performed and it did not show any major departures from the assumptions, which indicated that the model was appropriate for the data.

![FIGURE 1](image1.png) ![FIGURE 2](image2.png)

**FIGURE 1.** Projection of the independent variables on the factor plane [factor 1 (37.13%) compared with factor 2 (26.60%)]. PAT, number of patients per study; Time, treatment length.

**FIGURE 2.** Projection of the dependent variables on the factor plane [factor 1 (58.21%) compared with factor 2 (26.06%)]. TG, triacylglycerol; Chol, cholesterol; HDLC, HDL cholesterol; LDLC, LDL cholesterol.

### TABLE 7

<table>
<thead>
<tr>
<th>Variable</th>
<th>Δ%Cholesterol</th>
<th>Δ%Triacylglycerol</th>
<th>Δ%HDL cholesterol</th>
<th>Δ%LDL cholesterol</th>
<th>PCD1</th>
<th>PCD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients per study</td>
<td>0.069</td>
<td>0.003</td>
<td>−0.060</td>
<td>0.132</td>
<td>−0.078</td>
<td>−0.140</td>
</tr>
<tr>
<td>Dose</td>
<td>0.105</td>
<td>0.073</td>
<td>0.061</td>
<td>0.046</td>
<td>0.070</td>
<td>−0.008</td>
</tr>
<tr>
<td>Age</td>
<td>−0.028</td>
<td>0.001</td>
<td>−0.163</td>
<td>−0.067</td>
<td>0.018</td>
<td>−0.115</td>
</tr>
<tr>
<td>BMI</td>
<td>0.035</td>
<td>−0.099</td>
<td>0.011</td>
<td>0.051</td>
<td>−0.119</td>
<td>0.084</td>
</tr>
<tr>
<td>Treatment length</td>
<td>0.189</td>
<td>−0.040</td>
<td>0.041</td>
<td>0.261</td>
<td>0.201</td>
<td>0.325</td>
</tr>
<tr>
<td>PCI1</td>
<td>0.088</td>
<td>−0.049</td>
<td>−0.058</td>
<td>0.098</td>
<td>−0.132</td>
<td>−0.102</td>
</tr>
<tr>
<td>PCI2</td>
<td>−0.074</td>
<td>−0.016</td>
<td>0.006</td>
<td>−0.156</td>
<td>0.056</td>
<td>0.238</td>
</tr>
</tbody>
</table>

1 Δ, change.
2 P < 0.05.
3 P < 0.01.
DISCUSSION

Why submit the data obtained by this meta-analysis to multivariate statistical analysis?

It is impossible to use the simple ordering of information to reach the objective of this study. Multivariate analysis presents several alternative statistical procedures that permit a simplified structure of the data without relevant loss of information, which transforms an expressive number of original variables into a smaller number of new noncorrelated variables (15).

Regression models and univariate analysis have often been used in a statistical approach to a meta-analysis, which indicates important correlations between independent and dependent variables. However, many of these variables are correlated and could be substituted by principal components. Multivariate techniques are able to identify such correlations, taking into account a $p$ number of variables of interest, with the same weight and at the same time, expressing these correlations graphically. It can be said that techniques such as PCA for quantitative variables present a complete picture of the study, the visual

FIGURE 3. Projection of the studies and substudies on the factor plane produced by the independent variables (factor 1 compared with factor 2), where references 16–43 represent studies of phytosterols; 4, 7, and 44–61 represent studies of soluble fibers; and 10 and 62–90 represent studies of fatty acids. The letters following the reference numbers represent substudies.

FIGURE 4. Projection of the studies and substudies on the factor plane produced by the dependent variables (factor 1 compared with factor 2), where references 16–43 represent studies of phytosterols; 4, 7, and 44–61 represent studies of soluble fibers; and 10 and 62–90 represent studies of fatty acids. The letters following the reference numbers represent substudies.
significantly different, other studies, which take into account the net effect of the experimental group. In a meta-analysis, Bucher et al (92) investigated what information can be obtained from the reduction of factorial alterations observed in Figure 4 were obtained from a randomized experimental condition. The mixed-model analysis (Table 8) showed that PCD1 had a significant effect on both PCD1 and PCD2, and it is suggested that treatment length was the variable responsible for this effect (Table 7). That is, a shorter treatment length (PCD2 negative) reduces PCD1 and increases PCD2. Although the second principal component represents the most desirable outcome for blood lipoproteins, ie, a reduction in triacylglycerol followed by a reduction in cholesterol and LDL cholesterol, the variation promoted by the dietary intervention assessed in this study was better represented in the first and second principal components. In the latter case, soluble fibers showed an effect different from that of n-3 fatty acids and phytosterols.

The dietary interventions promoted a positive correlation between the percentage change in cholesterol, LDL cholesterol, and HDL cholesterol and an inverse correlation with the percentage change in triacylglycerol (Table 6). n-3 Fatty acids have a different effect on PCD2 compared with soluble fibers and phytosterols as a consequence of the different way in which these 3 bioactive substances act on human metabolism. Different mechanisms are responsible for the cholesterol-lowering effect of free and esterified phytosterols, such as competition for solubilization in dietary mixed micelles, cocrystallization with cholesterol to form insoluble mixed crystals, and interference with hydrolysis processes by lipases and cholesterol esterases (95). Evidence suggests that some soluble fibers bind bile acids or cholesterol during the intraluminal formation of micelles. The resulting reduction in the cholesterol content of liver cells leads to an up-regulation of the LDL receptors and thus an increased clearance of LDL cholesterol. Soluble fibers could also promote the inhibition of hepatic fatty acid synthesis by products of fermentation as short-chain fatty acids (94). The hyperlipidemia caused by n-3 fatty acid diets is well established and has been associated with various hepatic mechanisms such as increased fatty acid oxidation and inhibition of..
de novo fatty acid synthesis secondary to decreased fatty acid synthesis gene expression (96).

The percentage changes in cholesterol, LDL cholesterol, and HDL cholesterol were negatively correlated with the first principal component, whereas triacylglycerol made a positive contribution or that nutritional interventions that resulted in a reduction in the blood concentrations of triacylglycerol caused a considerable increase in LDL cholesterol, total cholesterol, and HDL cholesterol (Figures 2 and 4). This information should be printed on the labels of these functional foods because they can be purchased directly by consumers in supermarkets without medical supervision. In a study conducted in 6 hypertriglyceridemic patients supplemented with fish-oil concentrate, a 35% reduction in triacylglycerol was observed, which was accompanied by a 25% increase in LDL cholesterol, which suggested that n-3 fatty acids may enhance the propensity of VLDL cholesterol to be converted to LDL cholesterol (97). This could be a problem for those patients with modest elevations in triacylglycerol, in whom the elevation in LDL cholesterol impedes them from achieving their desired LDL-cholesterol concentration (8).

In summary, phytosterols and soluble fibers have a significant hypcholesterolemic effect, whereas n-3 fatty acids decreased triacylglycerol and increased total cholesterol, LDL cholesterol, and HDL cholesterol. The PCA and mixed models were able to show that this behavior is independent of dose, number of patients per study, age, and BMI but is associated with treatment length.

How should the results of this meta-analysis be used for the development of a functional food aimed at reducing the risk factors for coronary heart disease?

On the basis of the information generated by PCA and mixed models, the most adequate alternative for further studies should be the use of mixtures of the 3 bioactive substances to explore the maximum reduction of cholesterol, LDL cholesterol, and triacylglycerol with a maximum increase in HDL cholesterol. Other aspects of these 3 bioactive substances beside the hypolipidemic effect should be considered when developing function foods. For example, the major benefit of eating fiber-rich foods, including soluble fibers, may be a change in dietary pattern, resulting in a diet that is lower in saturated and trans unsaturated fats and cholesterol and higher in protective nutrients such as unsaturated fatty acids, minerals, folate, and antioxidant vitamins. Soluble fibers could promote slower absorption of macronutrients and increased satiety, which results in an overall lower energy intake (94). In addition, the contribution of n-3 fatty acids to the reduction in risk of coronary heart disease is mainly due to their effect at the vascular level. Eicosanoids synthesized from eicosapentaenoic acid are less potent in their ability to cause platelet aggregation or an inflammatory response than are corresponding eicosanoids derived from arachidonic acid (3, 83, 98, 99). Additional benefits of fish oils include improvements in endothelial function, better arterial elasticity, and modulation of inflammatory markers (100).

Conclusions

Although no health claims have been authorized on the basis of meta-analyses alone, they may be applied as supporting evidence for them. The multivariate statistical analysis applied in this study, PCA, showed correlations between 3 bioactive substances (n-3 fatty acids, soluble fibers, and phytosterols) and 4 blood biomarkers for coronary heart disease graphically. On the basis of these results, a more interesting proposal will be to develop further research that involves mixtures of these substances. The mixtures could be applied both for the purpose of developing new foods and for individual dietary planning, which will provide consumers with dietary alternatives capable of positively affecting plasma biomarkers and thus contribute to the control of coronary heart disease risk factors.

REFERENCES

marine-derived n-3 polysaturated fatty acids have differential effects on fasting and postprandial blood lipid concentrations and on the susceptibility of LDL to oxidative modification in moderately hyperlipidemic subjects. Am J Clin Nutr 2003;77:783–95.

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69. Tinker LF, Parks EJ, Behr SR, Scheeman BO, Davis PA. (n-3) fatty acid supplementation in moderately hypertriglyceridemic adults changes postprandial lipid and apolipoprotein B responses to a standardized test meal. J Nutr 1999;129:1126–34.

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A high-protein diet induces sustained reductions in appetite, ad libitum caloric intake, and body weight despite compensatory changes in diurnal plasma leptin and ghrelin concentrations

David S Weigle, Patricia A Breen, Colleen C Matthys, Holly S Callahan, Kaatje E Meeuws, Verna R Burden, and Jonathan Q Purnell

ABSTRACT
Background: Ad libitum, low-carbohydrate diets decrease caloric intake and cause weight loss. It is unclear whether these effects are due to the reduced carbohydrate content of such diets or to their associated increase in protein intake.
Objective: We tested the hypothesis that increasing the protein content while maintaining the carbohydrate content of the diet lowers body weight by decreasing appetite and spontaneous caloric intake.
Design: Appetite, caloric intake, body weight, and fat mass were measured in 19 subjects placed sequentially on the following diets: a weight-maintaining diet (15% protein, 35% fat, and 50% carbohydrate) for 2 wk, an isocaloric diet (30% protein, 20% fat, and 50% carbohydrate) for 2 wk, and an ad libitum diet (30% protein, 20% fat, and 50% carbohydrate) for 12 wk. Blood was sampled frequently at the end of each diet phase to measure the area under the plasma concentration versus time curve (AUC) for insulin, leptin, and ghrelin.
Results: Satiety was markedly increased with the isocaloric high-protein diet despite an unchanged leptin AUC. Mean (±SE) spontaneous energy intake decreased by 441 ± 63 kcal/d, body weight decreased by 4.9 ± 0.5 kg, and fat mass decreased by 3.7 ± 0.4 kg with the ad libitum, high-protein diet, despite a significantly decreased leptin AUC and increased ghrelin AUC.
Conclusions: An increase in dietary protein from 15% to 30% of energy at a constant carbohydrate intake produces a sustained decrease in ad libitum caloric intake that may be mediated by increased central nervous system leptin sensitivity and results in significant weight loss. This anorexic effect of protein may contribute to the weight loss produced by low-carbohydrate diets. Am J Clin Nutr 2005;82:41–8.

KEY WORDS Satiety, energy balance, adipose tissue, obesity, body composition, insulin

INTRODUCTION
The poor long-term outcome of energy-restricted diets for weight loss (1) has led to great interest in weight-reducing diets in which the macronutrient composition is altered but the caloric intake is not overtly specified. Both low-fat diets (2–4) and low-carbohydrate diets that are high in fat and protein (5–7) have been shown to cause a decrease in ad libitum caloric intake and significant weight loss in humans. Thus, it appears that diets with fat contents at opposite extremes have the same therapeutic result, despite evidence that excessive dietary fat intake promotes obesity (8, 9). This paradox could be explained if it is the high-protein content rather than the lower carbohydrate content of low-carbohydrate diets that offsets the deleterious effect of high fat intakes and results in weight loss.

Studies of macronutrient effects on energy balance are clouded by the inability to vary dietary protein, carbohydrate, and fat content independently of one another. In recently published studies of ad libitum, low-carbohydrate diets, experimental and control subjects consumed diets in which neither fat content nor protein content were held constant between groups (5–7). In the only published long-term study designed specifically to compare the effects of ad libitum diets of normal- and high-protein content, the fat content of the 2 diets was held constant (10). Thus, it could not be determined whether weight loss observed in the subjects who consumed the high-protein diet was due to the increase in dietary protein or the resulting decrease in dietary carbohydrate.

We undertook the present study to further evaluate the hypothesis that increasing the dietary protein content while maintaining the carbohydrate content lowers body weight by decreasing appetite and spontaneous caloric intake. This study was designed to complement a previous study in which the dietary fat content was lowered but the protein content was held constant (11). Subjects in both investigations served as their own controls and were studied under isocaloric intake and ad libitum feeding conditions. Plasma insulin, leptin, and ghrelin concentrations were measured frequently over 24-h periods to elucidate the mechanism of any observed changes in appetite or body composition. Our goal was to determine whether an increased protein intake...
TABLE 1
Subject characteristics at the time of enrollment

<table>
<thead>
<tr>
<th>Value (n = 3 M, 16 F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
</tr>
<tr>
<td>41 ± 11 (27–62)</td>
</tr>
<tr>
<td>Weight (kg)</td>
</tr>
<tr>
<td>72.0 ± 8.9 (56.1–88.3)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>26.2 ± 2.1 (22.5–30.1)</td>
</tr>
</tbody>
</table>

All values are x ± SD; range in parentheses.

confers some of the therapeutic benefits attributed to the currently popular low-carbohydrate diets.

SUBJECTS AND METHODS

Subjects

Nineteen healthy adults were recruited by newspaper and advertisements. Eleven subjects from the Seattle area were studied at the General Clinical Research Center (GCRC) of the University of Washington after approval of the protocol by the University of Washington Human Subjects Review Committee. Eight subjects from the Portland, OR, area were studied at the Oregon Health and Science University (OHSU) GCRC after approval of the protocol by the OHSU Human Subjects Review Committee. Characteristics of the subjects at the time of enrollment are summarized in Table 1. All subjects were weight-stable for ≥3 mo before enrollment and were at their lifetime maximal weight. Exclusion criteria included a body mass index (BMI; in kg/m²) ≥30, regular aerobic exercise (>30 min 3 times/wk), tobacco use, consumption of ≥2 alcoholic beverages/d, diabetes, chronic medical illness, or pregnancy. Prospective subjects were informed that this was not a weight-reduction study and were not enrolled if they expressed any expectation of losing weight.

The subjects provided informed written consent before enrollment.

Study protocol

After enrollment, subjects completed a 3-d food record and were interviewed by a GCRC dietitian. Individuals were excluded from the study at this point if they typically consumed a diet containing ≥55% or <35% of total calories from carbohydrate. Subjects were then placed on a baseline diet consisting of 35% of total daily energy as fat, 50% as carbohydrate, and 15% as protein. All meals were prepared in the Nutrition Research Kitchens of the GCRC at the University of Washington and consisted of typical foods found in a mixed American diet (12). A 3-d cycle of a standardized menu provided the required macronutrient distribution over the course of each day. Subjects visited the GCRC 2–3 times weekly to meet with a dietitian, be weighed, and pick up their food and appetite logs and make the same twice-weekly GCRC visits as described above. At each GCRC visit, the subjects returned their food and appetite logs and made the same weekly body-composition assessment by dual-energy X-ray absorptiometry scanning at visit CRC2.

After visit CRC2, the dietary macronutrient distribution remained fixed at 20% fat, 50% carbohydrate, and 30% protein; however, subjects were instructed to eat only as much of the diet as they wished (ad libitum phase). Specifically, they were told to eat when hungry, stop eating when satisfied, and avoid making any conscious effort to modify food intake, physical activity, or body weight. Three additional menu days were added to those of the second dietary period to provide a 6-d menu cycle. Sufficient food was provided on this ad libitum high-protein diet to allow subjects to consume up to 15% more than their weight-maintaining daily caloric intake. To decrease boredom and increase compliance with the diet, the subjects were allowed to eat one nonstudy meal and to consume up to 3 servings of alcoholic beverages in a 7-d period. They were also allowed substitutions for fruit and vegetables, depending on seasonal availability, and were provided with supplemental foods that matched the nutrient composition of the diet.

The subjects completed the same daily food logs, recorded the same appetite information, and made the same twice-weekly GCRC visits as described above. At each GCRC visit, the subjects returned their food and appetite logs and all uneaten food items from the previous visit. The GCRC nutrition staff weighed back all returned food items to determine actual daily calorie and macronutrient consumption. The subjects readmitted to the GCRC (visit CRC3) after 12 wk of ad libitum high-protein meal consumption. The study diet for the last day of the 2-wk baseline period, subjects were admitted to the GCRC for placement of an intravenous catheter (visit CRC1); the study diet for that day of the cycle was administered in 3 meals given at 0800, 1200, and 1730 with a snack at 2000. Blood was drawn into EDTA-coated tubes at 30-min intervals from 0800 to 2100 and then hourly until 0800 the next morning. Plasma was separated and stored at −70 °C. At 0730 on the second morning, supine resting metabolic rate (RMR) was measured by indirect calorimetry over a 30-min period with a ventilated hood connected to a metabolic cart with a model 29n Indirect Calorimeter (SensorMedics, Yorba Linda, CA) at the OHSU and with a TrueOne 2400 (Parvomedics Inc, Sandy, UT) at the University of Washington. Because of a period of equipment unavailability, RMR was measured in only 11 of the 19 study participants. Subjects were discharged from the GCRC after the 0800 blood drawing.

During the 2 wk immediately after visit CRC1, the subjects were placed on an isocaloric high-protein diet consisting of 20% fat, 50% carbohydrate, and 30% protein, with a 3-d cycle menu. Daily caloric intake was fixed at the level that would result in a stable weight with the baseline diet, and subjects were instructed to eat all food provided. The subjects continued to keep a daily food log, record appetite information, and visit the GCRC 2–3 times weekly to meet with a dietitian, be weighed, and pick up meals for the next 2 or 3 d. The subjects were readmitted to the GCRC on the last day of this 2-wk period (visit CRC2). The study diet for that day of the cycle was provided, blood was sampled, and RMR was measured as during visit CRC1. All subjects underwent body-composition assessment by dual-energy X-ray absorptiometry scanning at visit CRC2.

On the last day of the 2-wk baseline period, subjects were admitted to the GCRC for placement of an intravenous catheter (visit CRC1); the study diet for that day of the cycle was administered in 3 meals given at 0800, 1200, and 1730 with a snack at 2000. Blood was drawn into EDTA-coated tubes at 30-min intervals from 0800 to 2100 and then hourly until 0800 the next morning. Plasma was separated and stored at −70 °C. At 0730 on the second morning, supine resting metabolic rate (RMR) was measured by indirect calorimetry over a 30-min period with a ventilated hood connected to a metabolic cart with a model 29n Indirect Calorimeter (SensorMedics, Yorba Linda, CA) at the OHSU and with a TrueOne 2400 (Parvomedics Inc, Sandy, UT) at the University of Washington. Because of a period of equipment unavailability, RMR was measured in only 11 of the 19 study participants. Subjects were discharged from the GCRC after the 0800 blood drawing.

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Examples of the 15%-protein and 30%-protein diets are given in Table 2. The macronutrient composition of the diets was calculated by using the PRONUTRA database and is given in

TABLE 2

| Daily caloric intake was fixed at the level that would result in a stable weight with the baseline diet, and subjects were instructed to eat all food provided. The subjects continued to keep a daily food log, record appetite information, and visit the GCRC 2–3 times weekly to meet with a dietitian, be weighed, and pick up meals for the next 2 or 3 d. The subjects were readmitted to the GCRC on the last day of this 2-wk period (visit CRC2). The study diet for that day of the cycle was provided, blood was sampled, and RMR was measured as during visit CRC1. All subjects underwent body-composition assessment by dual-energy X-ray absorptiometry scanning at visit CRC2.

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Examples of the 15%-protein and 30%-protein diets are given in Table 2. The macronutrient composition of the diets was calculated by using the PRONUTRA database and is given in
Table 3. Total dietary fiber averaged 11.8 g/1000 kcal for the 15%-diet and 10.2 g/1000 kcal for the 30%-protein diets. Calcium intake averaged 450 mg/1000 kcal for the 15%-protein diet and 700 mg/1000 kcal for the 30%-protein diets. The average fatty acid composition of the 15%-protein diet as a percentage of total energy was 12.7% saturated, 11.5% monounsaturated, and 9.9% polyunsaturated; that of the 30%-protein diets was 7.6% saturated, 7.4% monounsaturated, and 3.9% polyunsaturated.

Hormone assays

Plasma insulin was measured with a double-antibody radioimmunoassay (14). The lower and upper detection limits were 2.2 and 300 μU/mL, respectively, and the intraassay CV was 7%. Leptin was measured with a commercially available radioimmunoassay (Linco Research, St Charles, MO) with lower and upper detection limits of 0.5 and 100 ng/mL, respectively, and an intraassay CV of 5%. All insulin and leptin samples from a single subject were run in duplicate in a single assay. Plasma immuno reactive ghrelin was measured with a commercially available radioimmunoassay (Phoenix Pharmaceuticals, Belmont, CA) with lower and upper detection limits of 80 and 2500 pg/mL, respectively; an intraassay CV of 8.7%; and an interassay CV of 14.6%. Because of the greater variability and lower capacity of the ghrelin assay, 24-h integrated plasma ghrelin concentrations were measured in pools of plasma created from the timed blood samples by combining 50 μL plasma from each blood sample drawn at 30-min intervals and 100 μL plasma from each blood sample drawn at 1-h intervals. Ghrelin concentrations in these pools were measured in duplicate in 2 separate assays that were normalized to one another by using internal controls as described previously (15).

Statistical analysis

Concentrations of all plasma hormones or fuel molecules, body composition, RMR, and caloric intake data are expressed as means ± SEs, unless noted otherwise. Nadirs of leptin time series data were defined as the average of the 2 lowest consecutive values, and peaks were defined as the average of the 2 highest consecutive values occurring over a 24-h period. Values for the 24-h integrated area under the curve (AUC) of plasma leptin, insulin, and glucose concentrations versus time were calculated above zero concentration by using the trapezoidal rule. In addition, AUC was calculated for plasma leptin concentrations minus the morning nadir value (AUC of the change in leptin). Ghrelin concentrations in pooled plasma were multiplied by 24 to calculate AUC ghrelin values. Within-subject comparisons among variables measured at visits CRC1, CRC2, and CRC3 were made by using repeated-measures analysis of variance with the Bonferroni correction applied to pairwise post hoc comparisons. Within-subject comparisons between variables measured only at visits CRC2 and CRC3 were made by using paired-samples t

Table 2. Menus for 1 d of the 15%-protein and 30%-protein diets

<table>
<thead>
<tr>
<th></th>
<th>15%-Protein diet</th>
<th>30%-Protein diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/2000 kcal</td>
<td>g/2000 kcal</td>
</tr>
<tr>
<td>Breakfast</td>
<td>Orange</td>
<td>Orange juice</td>
</tr>
<tr>
<td></td>
<td>Cream cheese (regular)</td>
<td>Egg Beaters egg whites</td>
</tr>
<tr>
<td></td>
<td>Plain bagel</td>
<td>Raisin bread</td>
</tr>
<tr>
<td></td>
<td>Reduced fat milk</td>
<td>Peanut butter</td>
</tr>
<tr>
<td>Lunch</td>
<td>Whole-wheat bread</td>
<td>Whole-wheat bread</td>
</tr>
<tr>
<td></td>
<td>Turkey breast</td>
<td>Turkey breast</td>
</tr>
<tr>
<td></td>
<td>Mayonnaise (regular)</td>
<td>“Light” Jarlsberg cheese</td>
</tr>
<tr>
<td></td>
<td>Leaf lettuce</td>
<td>Leaf lettuce</td>
</tr>
<tr>
<td>Dinner</td>
<td>Chicken fajita strips</td>
<td>Beef lasagna</td>
</tr>
<tr>
<td></td>
<td>Flour tortilla</td>
<td>Green beans</td>
</tr>
<tr>
<td></td>
<td>Green pepper strips</td>
<td>Lettuce</td>
</tr>
<tr>
<td></td>
<td>Onions</td>
<td>Tomatoes</td>
</tr>
<tr>
<td></td>
<td>Olive oil</td>
<td>Olive oil, for dressing</td>
</tr>
<tr>
<td></td>
<td>White rice</td>
<td>Wine vinegar, for dressing</td>
</tr>
<tr>
<td>Snack</td>
<td>Vanilla ice cream</td>
<td>Mandarin oranges, canned</td>
</tr>
<tr>
<td></td>
<td>Chocolate fudge sauce</td>
<td>Pineapple, canned</td>
</tr>
</tbody>
</table>

1 ConAgra Foods, Downers Grove, IL.
tests. Relations between pairs of variables were assessed by uni-
variate regression analysis with the use of a linear model. All
statistical analyses were carried out by using STATVIEW 5.0.1
software (SAS Institute Inc, Cary, NC).

RESULTS

We studied the consequences of a 15% increase in energy from
dietary protein in 19 subjects under weight-stable conditions
(isocaloric diets) during the first 4 wk of the protocol and during
active weight loss (ad libitum diet) in the final 12 wk of the
protocol. The dietary carbohydrate content was constant at 50%
of total daily caloric intake throughout the study. The subjects
successfully maintained a stable body weight during the isoca-
loric diet phase, with no significant change in mean weight
throughout the study. The meal-related changes in plasma insulin and glucose concentrations were as expected, as shown in Figure 5 and Figure 6, respectively. No significant differences were observed in fast-
ing plasma concentrations of free fatty acids, glucose, or insulin
measured during visit CRC1, CRC2, or CRC3 (Table 6). The
isocaloric high-protein diet led to significant increases in AUC
values for the 24-h insulin profiles obtained during CRC2 compared
with those obtained during visit CRC1. In contrast, 12 wk of ad
libitum high-protein diet consumption led to significant reduc-
tions in all of the variables characterizing the leptin profiles at
visit CRC3 (Figure 3). The percentage change in body fat mass
from visit CRC2 to visit CRC3 accounted for 32% of the vari-
ability in the percentage change in leptin AUC over this interval
(Figure 4).

Leptin pulse amplitude was assessed by calculating the differ-
ence between peak and nadir plasma leptin concentrations and
by calculating the AUC of the leptin profiles after subtracting
nadir leptin concentrations (AUC for the change in leptin). As
shown in Table 5, the peak minus nadir leptin concentration was
significantly lower at visit CRC3 than at visit CRC1 and was
lower at visit CRC2 than at visit CRC1 (P = 0.09). There was a
trend toward a reduction in the AUC for the change in leptin
during visits CRC2 and CRC3 relative to visit CRC1 (P = 0.15;
Figure 3).

The 24-h plasma leptin profiles measured during visits CRC1,
CRC2, and CRC3 are shown in Figure 3, and the variables
characterizing these profiles are summarized in Table 5. The
isocaloric high-protein diet led to no significant change in nadir
plasma concentrations, peak plasma concentrations, or AUC
values for the 24-h leptin profiles obtained during CRC2 compared
with those obtained during visit CRC1. In contrast, 12 wk of ad
libitum high-protein diet consumption led to significant reduc-
tions in all of the variables characterizing the leptin profiles at
visit CRC3 (Figure 3). The percentage change in body fat mass
from visit CRC2 to visit CRC3 accounted for 32% of the vari-
ability in the percentage change in leptin AUC over this interval
(Figure 4).
ghrelin assays that were tightly linked by means of multiple internal control samples. Ghrelin AUC values measured after 12 wk of ad libitum high-protein diet consumption during CRC3 were significantly greater than were the values measured during visit CRC1 (Table 6). Ghrelin AUC values measured during visit CRC2 did not differ significantly from those measured during visit CRC1.

**DISCUSSION**

We found that an increase in dietary protein content comparable with that observed in popular low-carbohydrate diets, but no reduction in dietary carbohydrate content, resulted in rapid losses of weight and body fat. This favorable change in body composition was due to a sustained decrease in appetite and ad libitum caloric intake. It is likely that a reduction in dietary fat by 15% of total energy contributed to weight loss in the present study. However, a larger 20% reduction in dietary fat with no change in the percentages of calories from protein produced less weight loss (3.7±0.6 compared with 4.9±0.5 kg; *P*<0.13) in our previous study (11). The protocol and 12-wk ad libitum diet periods were identical in both studies. These results suggest that substituting protein for fat in the diet may lead to greater weight loss than can be obtained by substituting carbohydrate for dietary fat. A larger study in which subjects are concurrently randomly assigned to both types of diets will be required to be certain of their relative ability to promote weight loss.

There are 2 mechanisms by which increased dietary protein intakes can promote a negative energy balance and loss of body fat. The first is the ability of dietary protein to increase energy expenditure. This is a small but significant increase that may depend on the relative proportion of animal and vegetable protein in the diet (16) and is partly due to greater diet-induced thermogenesis after protein consumption than after consumption of equal caloric loads of carbohydrate or fat (16–18). Increased dietary protein has also been shown to raise total daily energy expenditure in subjects at energy balance and to attenuate decreases in both sleeping metabolic rate and total daily energy expenditure in subjects following energy-restricted diets (19–21). We found that RMR, the major component of total daily energy expenditure, did not increase with the high-protein diets and that overall weight loss during ad libitum feeding was fully explained by the cumulative reduction in caloric intake. Thus, with the use of real food items and the natural feeding conditions used in the present study, increased thermogenesis did not appear to contribute significantly to the observed weight loss.

A more important mechanism by which dietary protein promotes weight loss appears to be its ability to produce greater satiety than do other macronutrients. This effect was shown in short-term feeding studies that used subjective appetite measures

<table>
<thead>
<tr>
<th>CRC1</th>
<th>CRC2</th>
<th>CRC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>72.1 ± 2.0a</td>
<td>72.0 ± 2.1a</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>—</td>
<td>24.1 ± 1.3a</td>
</tr>
<tr>
<td>Percentage body fat (%)</td>
<td>—</td>
<td>33.7 ± 1.5a</td>
</tr>
<tr>
<td>Energy intake (kcal/d)</td>
<td>2356 ± 80a</td>
<td>2325 ± 85a</td>
</tr>
<tr>
<td>Resting metabolic rate (kcal/d)</td>
<td>1542 ± 63a</td>
<td>1568 ± 58a</td>
</tr>
</tbody>
</table>

All values are $\bar{x} \pm SE$; $n = 19$ for all measurements except resting metabolic rate ($n = 11$). CRC, Clinical Research Center. Means with different superscript letters are significantly different, *P* < 0.05 [repeated-measures ANOVA with the Bonferroni correction applied to pairwise post hoc comparisons (weight, energy intake, and resting metabolic rate) or paired-samples *t* tests (fat mass and percentage body fat)].
or test meal consumption as endpoints (22–25). In most long-
term studies, the effect of increased protein consumption on
appetite was obscured by caloric restriction imposed on subjects
in addition to the altered macronutrient content of the diet (26–
29). Only one long-term, controlled ad libitum feeding study by
Astrup et al (10, 30) has been designed specifically to investigate
the effect of increased dietary protein content on spontaneous
energy intake and body composition. In this study, both the
reduction in caloric intake and magnitude of weight loss at 6 mo
in the subjects who consumed a 25% protein diet were signifi-
cantly greater than the values observed in subjects who con-
sumed a 12% protein diet (10). These differences persisted, but
were attenuated after consumption of the study diets for 12 mo
(30). Although the overall conclusion of this study agrees with
ours, no information was provided regarding the effects of the
diet on hunger, satiety, or circulating concentrations of hormones
known to be involved in body weight regulation.

Our subjects reported a marked increase in satiety with the
isocaloric high-protein diet despite an insignificant change in
leptin AUC between visit CRC1 and visit CRC2. Their ad libitum
energy intake after 12 wk of the high-protein diet remained 441 ±
63 kcal/d lower than baseline, despite a significant decrease in
leptin AUC between visit CRC2 and visit CRC3. This decrease
in spontaneous caloric intake was significantly greater (P =
0.04) than the 222 ± 81 kcal/d decrease noted at 12 wk in our
previous study of carbohydrate substitution for dietary fat at
constant protein intake (11). Taken together, these results sug-
gest that increased protein intake enhances the satiating effect of
circulating leptin in the central nervous system (CNS). Unlike
our previous study, ghrelin AUC values were significantly in-
creased after 12 wk of ad libitum high-protein intake (11). A
growing body of evidence suggests that an increase in circulating
ghrelin concentrations should increase appetite, thereby attenu-
ating the reductions in caloric intake and body weight that we
observed in the present study (31). The anorexic effect of dietary
protein, which may be due in part to increased CNS leptin sen-
sitivity, is apparently stronger than any orexigenic effect of in-
creased ghrelin concentrations accompanying weight loss with a
high-protein diet.

| Table 5 |
|------------------|------------------|------------------|
|                  | CRC1             | CRC2             | CRC3             |
| Nadir (ng/mL)    | 12.8 ± 2.2a      | 12.5 ± 1.9b      | 7.7 ± 1.1b       |
| Peak (ng/mL)     | 21.1 ± 3.2a      | 19.3 ± 2.6cb     | 13.8 ± 1.8b      |
| Peak − nadir (ng/mL) | 8.3 ± 1.2a      | 6.8 ± 0.88b      | 6.1 ± 0.9b       |
| AUC leptin (ng · 24 h/mL) | 402 ± 62a      | 375 ± 51a        | 259 ± 35b        |
| AUC for change in leptin (ng · 24 h/mL) | 94 ± 14a      | 75 ± 8a          | 74 ± 11a         |

1 All values are ± SE; n = 19. CRC, Clinical Research Center. Means with different superscript letters are significantly different, P < 0.05
(repeated-measures ANOVA with the Bonferroni correction applied to pairwise post hoc comparisons).
2 Calculated as described in Subjects and Methods.

Figure 4. Percentage change in the area under the plasma leptin con-
centration versus time curve (AUC) in 19 healthy subjects between visits 2
and 3 to the General Clinical Research Center (CRC2 and CRC3) plotted
against the percentage change in body fat mass over the corresponding time
period. The line indicates the best least-squares fit to the data: y = 1.28x
−9.35 (r = 0.568, P < 0.01).

Figure 5. Mean 24-h plasma insulin concentrations in 19 healthy
subjects during visits 1 (□), 2 (○), and 3 (∆) to the General Clinical Research
Center. The arrows indicate the times at which the major meals (B, breakfast;
L, lunch; D, dinner) were consumed. The error bars were omitted for clarity.
Havel et al (32) reported that substitution of carbohydrate for dietary fat increased the diurnal circulating leptin pulse amplitude (peak minus nadir plasma leptin concentration). These authors speculated that, as for other endocrine systems (33, 34), the CNS might interpret an increase in leptin pulse amplitude as a signal calling for a decrease in appetite independently from any change in the integrated circulating leptin concentration (32). Our data directly address this hypothesis because we observed a decrease in leptin pulse amplitude between visit CRC1 and visit CRC2 (decreased peak minus nadir plasma leptin concentration and decreased AUC of the change in leptin) without a significant change in integrated circulating leptin concentration (leptin AUC). The subjects reported a marked increase in satiety despite this isolated reduction in leptin pulse amplitude. These data suggest that if diurnal leptin pulse amplitude is a signal regulating energy balance, it is less important than the putative change in CNS leptin sensitivity observed in the present study.

We found insulin AUC to be significantly higher at visit CRC2 than at visit CRC1, which possibly reflects the better ability of protein than of fat, which it was isocalorically substituted for, to stimulate insulin secretion (35). Because insulin appears to act synergistically with leptin in the hypothalamus (36), this increase in insulin AUC may have contributed to the increased satiety observed with the isocaloric high-protein diet. The decrease in insulin AUC to baseline values after 12 wk of the ad libitum high-protein diet most likely reflects a decrease in the stimulus for insulin secretion resulting from the overall decrease in energy intake by this point in the study.

In conclusion, a 15% increase in energy from dietary protein at constant carbohydrate intake produces a sustained decrease in ad libitum caloric intake that may be mediated by increased CNS leptin sensitivity and results in clinically significant weight loss. This salutary effect of protein may help to explain the paradoxical weight loss observed in subjects placed on low-carbohydrate diets, because an increase in protein intake accompanies the high fat content of such diets (5–7). Our results suggest that less emphasis should be placed on carbohydrate restriction without regard for concomitant increases in dietary fat. Replacing a portion of dietary fat with protein may result in weight loss comparable with that reported with low-carbohydrate diets while minimizing the adverse long-term effects of increased dietary fat. However, further study of the effects of dietary protein intake on renal function and calcium balance will be required before high-protein diets can be widely recommended for weight loss.

We thank Pamela Yang, Holly Edelbrock, Heidi Johnson, Heather Callahan, and Alison Shircliff for their expert technical assistance.

DSW was responsible for the experimental design, data analysis, and writing of the manuscript. PAB was responsible for the subject recruitment, data collection, and data analysis. CCM was responsible for the experimental design and diet design. HSC, KEM, and VRB were responsible for the diet design, subject screening, and data analysis. JQP was responsible for the experimental design and data analysis. No author had any personal or financial relation with the agencies funding this research.

REFERENCES


FIGURE 6. Mean 24-h plasma glucose concentrations in 19 healthy subjects during visits 1 (○), 2 (●), and 3 (△) to the General Clinical Research Center. The arrows indicate the times at which the major meals (B, breakfast; L, lunch; D, dinner) were consumed. The error bars were omitted for clarity.

TABLE 6
Plasma fuel molecule, insulin, and ghrelin data obtained during the final 24-h periods of the weight-maintaining 15%-protein diet (visit CRC1), the isocaloric weight-maintaining 30%-protein diet (visit CRC2), and the ad libitum 30%-protein diet (visit CRC3)∗

<table>
<thead>
<tr>
<th></th>
<th>CRC1</th>
<th>CRC2</th>
<th>CRC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting free fatty acids (mEq/L)</td>
<td>0.581 ± 0.037</td>
<td>0.571 ± 0.028</td>
<td>0.644 ± 0.034</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>86.2 ± 2.0</td>
<td>89.1 ± 1.8</td>
<td>85.9 ± 2.3</td>
</tr>
<tr>
<td>AUC glucose (mg · 24 h/dL)</td>
<td>2360 ± 31</td>
<td>2414 ± 27</td>
<td>2339 ± 46</td>
</tr>
<tr>
<td>Fasting insulin (μU/mL)</td>
<td>10.8 ± 1.0</td>
<td>12.0 ± 1.3</td>
<td>10.5 ± 1.1</td>
</tr>
<tr>
<td>AUC insulin (μU · 24 h/mL)</td>
<td>762 ± 47a</td>
<td>875 ± 62b</td>
<td>717 ± 38a</td>
</tr>
<tr>
<td>AUC ghrelin (pg · 24 h/mL)</td>
<td>13,979 ± 1072a</td>
<td>14,640 ± 1124ab</td>
<td>15,456 ± 1173b</td>
</tr>
</tbody>
</table>

∗ All values are x ± SE; n = 19. CRC, Clinical Research Center. Means with different superscript letters are significantly different, P < 0.05 (repeated-measures ANOVA with the Bonferroni correction applied to pairwise post hoc comparisons).
Bioelectrical impedance analysis: population reference values for phase angle by age and sex¹⁻³

Maria Cristina G Barbosa-Silva, Aluíso JD Barros, Jack Wang, Steven B Heymsfield, and Richard N Pierson Jr

ABSTRACT
Background: Phase angle is an indicator based on reactance and resistance obtained from bioelectrical impedance analysis (BIA). Although its biological meaning is still not clear, phase angle appears to have an important prognostic role.

Objective: The aim of this study was to estimate population averages and SDs of phase angle that can be used as reference values.

Design: BIA and other methods used to evaluate body composition, including hydrodensitometry and total body water, were completed in 1967 healthy adults aged 18–94 y. Phase angle was calculated directly from body resistance and reactance, and fat mass (FM) was estimated from the combination of weight, hydrodensitometry, and total body water by using the 3-compartment Siri equation. Phase angle values were compared across categories of sex, age, body mass index (BMI), and percentage FM.

Results: Phase angle was significantly (P < 0.001) smaller in women than in men and was lower with greater age (P < 0.001). Phase angle increased with an increase in BMI and was significantly inversely associated with percentage fat in men. Phase angle was significantly predicted from sex, age, BMI, and percentage FM in multiple regression models.

Conclusions: Phase angle differs across categories of sex, age, BMI, and percentage fat. These reference values can serve as a basis for phase angle evaluations in the clinical setting. Am J Clin Nutr 2005;82:49–52.

KEY WORDS Bioelectrical impedance analysis, phase angle, nutritional assessment, body composition, diagnostic methods

INTRODUCTION

Bioelectrical impedance analysis (BIA) is a noninvasive, inexpensive, and portable method that has been used mainly for body-composition analysis over the past decade. However, BIA does not measure body composition directly. It measures 2 bioelectrical parameters: body resistance and reactance. Resistance is the opposition offered by the body to the flow of an alternating electrical current, and it is inversely related to the water and electrolyte content of tissue. Reactance is related to the capacitance properties of the cell membrane, and variations can occur depending on its integrity, function, and composition (1).

BIA is considered to be a statistically derived fat-estimation method, because it depends on a regression analysis between impedance and a reference method for the development of a prediction formula (2). Many prediction equations are available to estimate body compartments as a function of resistance, reactance, anthropometric variables (weight and height), sex, and age. Prediction equations are only valid for the specific population they are developed for, which makes these equations inappropriate in clinical situations. Patients who are malnourished, who are critically ill, and who have eating disorders have a fluid imbalance; therefore, the constant hydration of lean body mass may not be acceptable (3).

Phase angle is a derived measure obtained from the relation between the direct measures of resistance and reactance (4). Phase angle is calculated directly from reactance and resistance:

\[ \text{Phase angle} = \arctan(\text{reactance}/\text{resistance}) \times 180°/\pi \]  

(1)

Its biological meaning and pathogenic effects are not completely understood. Phase angle has been interpreted as an indicator of membrane integrity and water distribution between the intra- and extracellular spaces (4). Phase angle has also been used to predict body cell mass (5, 6); for this reason, it has also been used as a nutritional indicator in adults and children (6, 7).

Some authors have studied the role of phase angle as a prognostic indicator. A positive association was shown between phase angle and survival in patients with HIV-positive AIDS (4, 8), with lung cancer (9), undergoing hemodialysis (5, 7), and who are critically ill (10, 11). These authors suggested that phase angle could be an important tool for evaluating clinical outcome or for monitoring disease progression and may be superior to other serum or anthropometric nutritional indicators.

The lack of phase angle reference values has limited its use in clinical and epidemiologic situations. Such values are needed to properly assess individual deviations in relation to the population average and to analyze the influence of phase angle on various outcomes within epidemiologic studies. We conducted the present study to understand the relation between phase angle and such variables as sex, age, race, and body-composition indicators [eg, body mass index (BMI) and percentage fat]. We also estimated population averages and SDs for phase angle to serve as

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² Supported by CAPES, Ministry of Education, Brazil, who partially funded this research through its scholarship program.
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reference values. With these reference values, it is possible to standardize individual values and to make comparisons between different age and sex groups in clinical or population studies.

SUBJECTS AND METHODS

Between 1986 and 1999, a study to evaluate body composition was performed at the St Luke’s–Roosevelt Hospital Center in 1967 healthy adults aged 18–94 y, who were recruited from hospital staff and the local area. All subjects were fully informed about the study objectives and methods and were asked to sign a written consent form. The Institutional Review Board of St Luke’s–Roosevelt Hospital approved the study.

The subjects were studied after fasting for ≥8 h. Several body-composition tests were performed, such as hydrodensitometry and total body water (TBW). Of direct interest to the present analysis, body weight (BW) was measured to the nearest 0.1 kg with a Weight-Tronix Scale (Scale Electronics Development, New York, NY) while each subject was wearing a hospital gown, underwear, and no shoes; height was measured to the nearest 0.1 cm with a wall-mounted stadiometer (Holtain Ltd, Crosswell, United Kingdom). BMI (kg/m²) was calculated as body weight/height squared. BIA was performed with the use of an RJL instrument (model 101; RJL Systems, Mt Clemens, MI), which applies an 800-KHz current at a frequency of 50 KHz. The measurements were performed under a strict standardization of the procedure, according to the National Institutes of Health (12). The subjects were in a supine position 5 min before the measurement, which was performed under a thermoneutral environment of 25 °C. Phase angle was calculated as previously described (1).

Fat mass (FM) was estimated by using the three-compartment Siri equation:

\[ FM = 2.1 \times \text{body volume} - 0.8 \times \text{TBW} - 1.3 \times \text{BW} \]  

and %FM was estimated as

\[ \%FM = \left(\frac{FM}{\text{weight}}\right) \times 100 \]  

Total body water was obtained from tritium space (³H₂O; in L) and corrected for nonaqueous hydrogen exchange. The details about these body-composition methods (TBW and hydrodensitometry) are described in detail elsewhere (2).

The statistical analyses were performed using STATA 6.0 (Stata Corporation, College Station, TX) (13). The correlations between phase angle and the other variables were estimated. The crude effect of sex, race, age, BMI, and %FM on phase angle was assessed by comparing the means of the first 2 variables (t test and ANOVA, respectively) and by using the correlation coefficients for the last 3 variables. A multiple linear regression analysis was used to adjust the effects of the variables and to identify those variables that were independently associated with phase angle. On the basis of these results, we could identify the smallest set of variables that explained most of the observed variability, so that reference values could be calculated for the smallest number of subgroups. The usual significance level of 5% was used for all tests.

RESULTS

The age, weight, height, and BMI of the 1967 study subjects are presented in Table 1; 46% of the subjects were white, 22% were African American, 14% were Asian, and 18% were Hispanic or of another race. The women (58%) were significantly older than the men. The mean BMI was 25.9; and no significant difference was found between the women and the men.

Phase angle was significantly larger in the men than in the women (7.48 ± 1.10° and 6.53± 1.01°, respectively; P < 0.001). A comparison of phase angle by race showed a significant difference in crude analysis (P < 0.001): 6.55 ± 1.1° for Asians, 6.82 ± 1.13° for whites, 7.00 ± 1.01° for multiracial subjects, 7.21 ± 1.19° for African Americans, 7.33 ± 1.13° for Hispanics, and 7.45 ± 0.98° for other races.

Phase angle showed a positive correlation with BMI (R² = 0.17) and a negative correlation with age and %FM (R² = −0.49 and −0.32, respectively); all correlations were significant (P < 0.001).

The final regression model obtained was rather complex and explained almost one-half of the observed variance in phase angle (R² = 0.49). After age and sex were controlled for, race was no longer significant, which suggested that the crude association was due to confounding. Sex, age, BMI, and %FM remained associated with phase angle, including the interactions of sex with age and of BMI with %FM. However, for sex and age it was possible to achieve 82% of the variability explained by the full model (0.40 out of 0.49).

Because BMI was significantly associated with phase angle in the previous analysis, it was important to check whether the distribution of this variable in our sample was similar to its distribution in the population. We thus compared the mean BMIs, by sex and age, with the mean BMIs published by Flegal and Triano (14) with the use of population-based data from the third National Health and Nutrition Examination Survey (NHANES III). Some differences were found: men and women from the study conducted in New York had a BMI lower than that of the NHANES III value, especially those aged >50 y. (The largest mean differences in BMI were 1.7 in men and 2.3 in women.) To correct for this difference, phase angle values were adjusted by NHANES III BMI means for each age and sex category. Mean differences of 0.03 and 0.04° were found between the original and adjusted values for women and men, respectively. The largest differences were found in persons aged >70 y: −0.09° (−1.5%) in women and −0.07° (−1.1%) in men. The corrections were of no clinical relevance, and the adjustment for BMI was abandoned.

Given that sex and age accounted for most of the phase angle variability explained by available variables and that BMI and %FM are not always available in clinical situations (eg, for bedridden patients), phase angle reference values were estimated for the subgroups generated by sex and age only.

TABLE 1

<table>
<thead>
<tr>
<th>Age, weight, height, and BMI of the study subjects²</th>
</tr>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Men (n = 832)</td>
</tr>
<tr>
<td>Women (n = 1135)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
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<tr>
<td>46.3 ± 18.3</td>
</tr>
<tr>
<td>48.1 ± 17.7</td>
</tr>
<tr>
<td>0.02</td>
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<tr>
<td>Weight (kg)</td>
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<tr>
<td>77.5 ± 14.7</td>
</tr>
<tr>
<td>67.9 ± 17.5</td>
</tr>
<tr>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Height (cm)</td>
</tr>
<tr>
<td>173.6 ± 7.6</td>
</tr>
<tr>
<td>161.5 ± 7.0</td>
</tr>
<tr>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>25.6 ± 4.2</td>
</tr>
<tr>
<td>26.0 ± 6.4</td>
</tr>
<tr>
<td>0.1</td>
</tr>
</tbody>
</table>

1 All values are x ± SD.
2 t test.
The distribution of phase angle was fairly normal in our data. Mean (±SD) phase angles, and 5th and 95th percentiles, are shown by age and sex in Table 2. The overall mean phase angle mean was 6.93 ± 1.15°; 7.48 ± 1.10° for men, and 6.53 ± 1.01° for women. Phase angle was significantly greater in the men than in the women in all age categories. There was a significant and decreasing linear trend in phase angle with age, in both sexes. Phase angle decreased from 7.90° (youngest group) to 6.19° (oldest group) in men and from 7.04° (youngest group) to 5.64° (oldest group) in women.

### Discussion

Phase angle has been reported to be a prognostic tool in various clinical situations, such as HIV (4, 8), bacteremia (15), cirrhosis of the liver (16), renal disease (5, 17–19), pulmonary tuberculosis (20), and cancer (9, 21). Despite this, relatively little is known about reference values for phase angle in healthy populations. The objective of this study was to obtain phase angle values in a sample of healthy subjects who were volunteers in other body-composition studies. This fact enabled us to study not only phase angles but also the relation of phase angles to other characteristics of body composition, such as body fat measured by using reference methods.

Phase angle can be calculated as the arc-tangent of the ratio of reactance to resistance and then converted to degrees. Some authors have used a simplified equation (phase angle = reactance/resistance; converted to degrees) to obtain its value. Although not strictly correct, the simplified equation gives similar results because the ratio between reactance and resistance results in very small values (from 0.06 to 0.2 in our sample). In this situation, the arc-tangent returned a similar value, but this would not have happened if the values were larger.

The high inverse correlation with age and positive correlation with BMI were also found by Dittmar (22). The finding of a higher phase angle in persons with a higher BMI is not surprising. Phase angle is directly related to cell membranes (amount and functional status), which are what reactance stands for. Persons with higher BMIs have more cells (fat or muscle cells), and this results in higher phase angle values.

The age- and sex-related differences found in our study were not found in some previous studies. Baumgartner et al (1), in the first study of phase angle and body composition, found no significant difference in phase angle values between sex and age groups. Selberg and Selberg (16) also found no significant difference in phase angle values by sex in healthy subjects, probably because of their very small sample size (74 adults and 48 subjects aged <18 y in Baumgartner et al’s study and 50 subjects in Selberg and Selberg’s study) and consequent lack of power. This difference, however, was found in larger studies of healthy adults (23, 24) and in a hemodialysis population (25). Buffa et al (26) also showed a significant decrease in phase angle with age in healthy elderly subjects, and Kyle et al (27) found the same age and sex differences in 2740 healthy adults.

The decrease in phase angle values with increasing age may suggest that phase angle is an indicator of function and general health, not only an indicator of body composition or nutritional status. The phase angle values found in a hemodialysis population were clearly smaller than those found in our healthy sample (median: 5.16° in men and 4.01° in women) (25). In the same study, the presence of diabetes resulted in phase angle values that were even smaller. A mean phase angle of 4.57° was found in lung cancer patients, and the survival of patients with a phase angle smaller than this value was significantly shorter (9). The use of standardized values found in our study makes possible the individual comparison of healthy and sick people with its age- and sex-specific phase angle mean. This approach is more likely to indicate a high-risk situation than is the comparison of individual values with the overall mean phase angle.

A study conducted in a Swiss population of healthy subjects was designed to determine reference values for fat-free mass, FM, and %FM obtained from BIA (23). In the Swiss population, phase angle values were smaller than those found in the present study (10.5% in men and 7.7% in women). Although the prevalences of overweight and obesity were lower in the Swiss study than in the US population in the present study, phase angle values remained smaller even after adjustment for BMI and %FM. This may suggest that phase angle, as other anthropometric variables, may have reference values that are specific to each population. Further studies are necessary to show how phase angle differs between different populations and whether they vary with the bioimpedance device used.

Once the sample was obtained from the subjects, we needed to know whether it could be considered representative of the US population. The adjustment for differences in the BMI distribution in the NHANES III data presented no clinically relevant effect on age- and sex-specific phase angle values. We are confident that our results can be used as reference values for the US population.
population and possibly for other populations with similar body composition. However, the reference values for the youngest group (18–20 y of age) in our study should be used with caution because of the small sample size of each sex in this group.

Because phase angles differ by age and sex, it becomes difficult to compare values across populations of different sexes and of different age groups. One way to make such values comparable, regardless of age and sex, is to standardize them, as is commonly done with nutritional status (e.g., weight is standardized for age and sex and transformed into a z score). Standardized phase angles for specific age and sex groups can be obtained by dividing mean age- and sex-specific phase angles by their SDs. Standardized phase angles have a mean of 0 and an SD of 1 for everyone and are comparable regardless of age and sex.

The prognostic role of phase angle is easier to assess if standardized values are used. Standardized phase angles on the positive side of the scale (i.e., values greater than the mean) are expected for healthy subjects. Sick individuals (e.g., cancer patients) are expected to have negative standardized phase angles (i.e., values lower than the mean), which become increasingly lower with a worsening prognosis. The use of standardized phase angles is likely to produce better results than is the use of a single population reference value for identifying high-risk persons.

In summary, we showed that phase angle changes with sex and age. Its dependence on body composition is complex, being determined by BMI, %FM, and their interaction. The age- and sex-specific means and SDs presented in this study make it possible to calculate standardized phase angle values that make comparisons across subjects possible, even when the age and sex of the population vary widely. Also, studies of the prognostic value of phase angle in various subject groups—such as surgical, cancer, and intensive care patients—will now have access to a single set of reference values. Furthermore, cutoffs determined to identify high-risk subjects, based on standardized phase angles, will not depend on the age and sex structure of the studied samples.

We thank the Obesity Research Center (St Luke’s–Roosevelt Hospital Center), especially Frederick Rubiano (Human Body Composition Laboratory Supervisor) and Dana Kotler. MCGB-S proposed the idea of the paper and had primary responsibility for the data analysis and the writing of the manuscript. AJDB helped choose the methodologic approach and helped with the data analysis and the writing of the manuscript. JW, SBH, and RNP Jr designed the experiment, conducted the study from which the data originated (body-composition studies), actively participated in the data analysis, and reviewed the manuscript. No conflicts of interest were declared.

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Body composition and mortality in chronic obstructive pulmonary disease\textsuperscript{1,2}

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ABSTRACT

Background: Survival studies have consistently shown significantly greater mortality rates in underweight and normal-weight patients with chronic obstructive pulmonary disease (COPD) than in overweight and obese COPD patients.

Objective: To compare the contributions of low fat-free mass and low fat mass to mortality, we assessed the association between body composition and mortality in COPD.

Design: We studied 412 patients with moderate-to-severe COPD [Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) stages II–IV, forced expiratory volume in 1 s of 36 ± 14% of predicted (range: 19–70%)]. Body composition was assessed by using single-frequency bioelectrical impedance. Body mass index, fat-free mass index, fat mass index, and skeletal muscle index were calculated and related to recently developed reference values. COPD patients were stratified into defined categories of tissue-depletion pattern. Overall mortality was assessed at the end of follow-up.

Results: Semistarvation and muscle atrophy were equally distributed among disease stages, but the highest prevalence of cachexia was seen in GOLD stage IV. Forty-six percent of the patients (n = 189) died during a maximum follow-up of 5 y. Cox regression models, with and without adjustment for disease severity, showed that fat-free mass index (relative risk: 0.90; 95% CI: 0.84, 0.96; \(P = 0.003\)) was an independent predictor of survival, but fat mass index was not. Kaplan-Meier and Cox regression plots for cachexia and muscle atrophy did not differ significantly.

Conclusions: Fat-free mass is an independent predictor of mortality irrespective of fat mass. This study supports the inclusion of body composition assessment as a systemic marker of disease severity in COPD staging. Am J Clin Nutr 2005;82:53–9.

KEY WORDS Mortality, chronic obstructive pulmonary disease, COPD, body composition, muscle mass, lung function

INTRODUCTION

Survival studies in selected groups of patients with chronic obstructive pulmonary obstruction (COPD) and in population-based studies have consistently shown higher COPD-related mortality rates in underweight and normal-weight patients than in overweight and even obese patients (1–3). This relation is different from the U-shaped survival curve that is commonly seen for body mass index (BMI) in large population studies (reviewed in 4). We hypothesized that this discrepancy might be explained by specific adverse effects of an excess loss of metabolically and functionally active fat-free mass (FFM) on mortality in chronic disease that are not seen with BMI. This increased mortality risk in COPD might be due to direct effects on lung function (5) or adverse effects of the loss of FFM on skeletal muscle strength (6), exercise capacity (7, 8), and health status (9) that may increase the frequency or severity of acute exacerbations of the disease. Furthermore, a recent study showed that a small midthigh cross-sectional area, as measured with computed tomography scan, was associated with increased mortality risk during a 3-y follow-up (10). In the same study, midthigh cross-sectional area could not be estimated by using anthropometric measurements. Bioelectrical impedance analysis (BIA) is an easy, safe, noninvasive, and convenient method of measuring the lean and fat body compartments (11). In addition, it has been validated extensively in COPD and other chronic wasting conditions, which have shown high correlations between FFM measured by BIA and that measured by reference methods such as magnetic resonance imaging (12) or deuterium dilution (13). The objective of this study was to compare the effects of low FFM and low fat mass (FM) assessed by BIA on mortality in COPD patients.

SUBJECTS AND METHODS

Subjects

Data were collected from 412 clinically stable COPD patients [Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) stages II–IV] who were screened for admission to a clinical pulmonary rehabilitation program between 1 January 1988 and 30 December 1991. This group was selected because it was a homogeneous group of clinically stable patients from the southern part of the Netherlands and because none of the patients had undergone interventions that might modulate body composition, eg, nutritional intervention, intensive exercise training, or muscle strength training, before screening and during the total follow-up of 5 y. Such interventions currently are common in the Netherlands as part of an integrated COPD management approach. Patients with unstable disease or other confounding conditions such as type 1 diabetes, cardiovascular disease, thyroid

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disease, or chronic inflammatory bowel disease were excluded. All baseline measurements were taken within 1 wk of admission to the rehabilitation center.

Lung function

Lung function testing included spirometry and resting arterial blood gases. Forced expiratory volume in 1 s (FEV₁) and inspiratory vital capacity (IVC) were measured with a wet spirometer by using the highest value of ≥3 acceptable spirometric maneuvers. Prebronchodilation and postbronchodilation FEV₁ and IVC were expressed as a percentage of the reference values (14). Arterial blood gases were drawn by puncture of the at-rest brachial artery while the subjects breathed room air. Arterial oxygen tension (PaO₂) and arterial carbon dioxide tension (PaCO₂) were analyzed with the use of a blood gas analyzer (ABL 330; Radiometer, Copenhagen, Denmark).

Body composition

Body height was measured to the nearest 0.5 cm while the subjects were barefoot and standing (WM 715; Lameris, Breukelen, Netherlands). Body weight was measured on a calibrated beam scale (model 708; Seca, Hamburg, Germany). Body composition was assessed by using single-frequency BIA (50 Hz; Xitron Technologies, San Diego, CA). All measurements were performed by the same trained dietitian at a standardized time after breakfast. FFM was calculated by using disease-specific equations (13). FM was calculated as total body weight minus FFM. A prerequisite of the use of BIA is that the equations used to transform the measured resistance into FFM or total body water are adapted to the individuals measured and have been tested for validity in the populations for which they are intended. In these conditions, BIA measurements are accurate and comparable to other techniques used to assess body composition. BIA has been extensively validated and evaluated in patients with COPD. The equations used were validated against deuterium X-ray absorptiometry (15, 16). The skeletal muscle index (SMI) was determined according to the equations used by Janssen et al (12).

Follow-up

Mortality was assessed on 31 January 1993. Patients were followed for 2–5 y or until death, whichever came first. Mortality was assessed as overall mortality due to all causes.

Statistical analysis

BMI, FFM index (FFMI), and FM index (FMI) were calculated by dividing body weight (in kg), FFM, and FM, respectively, by height (in m) squared to adjust for body surface area. Patients were stratified by body composition into 4 categories, as follows. Patients in category 1 (cachexia) had BMI <21 and FFMI <16 (men) or <15 (women); patients in category 2 (semistarvation) had BMI <21 and FFMI ≥16 (men) or ≥15 (women); patients in category 3 (muscle atrophy) had BMI ≥21 and FFMI <16 (men) or <15 (women); and patients in category 4 (no impairment) had BMI ≥21 and FFMI ≥16 (men) or ≥15 (women). The cutoff for FFMI is based on the linear association between FFM and body weight in normal-weight to underweight COPD patients as described by Baarends et al (8). In earlier publications, our group showed that these cutoffs are discriminative for exercise capacity (7) and health status (17). FFMI and FMI were compared with the sex-specific percentiles reported by Schutz et al (18). As an additional characterization, the SMI was measured, as proposed by Janssen et al, for a comparison of the total study population as well as the subgroups with results from the third National Health and Nutrition Examination Survey (NHANES III, 1988–1994) (19). The percentage of patients theoretically at risk for physical disability was assessed by using the cutoffs for physical disability risk set by Janssen et al. Subjects from NHANES III were classified as physically disabled (ie, having difficulty performing activities of daily living) if they answered “yes” to either or both of the following questions: 1) “Because of any impairment or health problem, do you need the help of other persons with personal care needs, such as eating, bathing, dressing, or getting around at home?” and 2) “Because of any impairment or health problem, do you need the help of other persons in handling routine needs, such as everyday household chores, doing necessary business, shopping, or getting around for other purposes?”

In the study by Janssen et al (19), the cutoffs for severe physical disability risk were set at SMI ≤5.75 (women) or ≤8.50 (men), and those for moderate physical disability risk were set at SMI 6.75 and >5.75 (women) or ≤10.75 and >8.50 (men).

Results are presented as mean (±SD) for all variables that were normally distributed. Univariate analysis was performed by using the Kaplan-Meier method. A log-rank chi-square test for comparing survival between groups was used to analyze the association between depletion pattern and survival. The Cox proportional hazards model was used to quantify the relation between mortality and body composition (ie, FFMI and FMI), age, sex (0 = women, 1 = men), disease severity [PaO₂, PaCO₂, and FEV₁ (% of predicted) as continuous variables], and GOLD classification (as a categorized variable). Lung function criteria that defined the GOLD stages were normal lung function, GOLD 0 (at risk); the ratio of FEV₁ to IVC <70% and FEV₁ ≥80%, GOLD I (mild); FEV₁/IVC <70% and FEV₁ <80% and ≥50%, GOLD II (moderate); FEV₁/IVC <70% and FEV₁ <50% and ≥30%, GOLD III (severe); and FEV₁/IVC <70% and FEV₁ <30% or FEV₁ <50% and PaO₂ <8.0 kPa, GOLD IV (very severe).

The relative risk (RR) corresponding to a risk factor in this model is the exponential of the regression coefficient. The differences in distribution of body composition categories in GOLD stages II–IV were tested by using the chi-square test.

We performed all analyses with and without inclusion of the variable smoking (0 = no smoking or former smoking, 1 = current smoking). However, the cumulative pack-year exposure was high in all patients, and status as a former smoker was not objectively verified (eg, by cotinine measurements in saliva). Therefore, smoking was not associated with mortality in these subjects, and we decided to report only the results without this variable in the model.

A two-sided value of P < 0.05 was considered significant. Baseline comparisons between groups stratified by disease severity or body composition were performed by using an unpaired Student’s t test with Bonferroni correction for multiple comparisons. Data were analyzed by using SPSS for WINDOWS statistical software (version 11.0; SPSS Inc, Chicago, IL).
TABLE 1
Patient characteristics

<table>
<thead>
<tr>
<th>Category</th>
<th>Percentage of patients</th>
<th>FFMI</th>
<th>Percentile</th>
<th>FMI</th>
<th>Percentile</th>
<th>SMI</th>
<th>Moderate</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>kg/m²</td>
<td></td>
<td>kg/m²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1: Cachexia (n = 92)</td>
<td>29</td>
<td>14.3</td>
<td>1.26</td>
<td>&lt;5th</td>
<td>4.4</td>
<td>1.3</td>
<td>&lt;50th</td>
<td>7.8</td>
</tr>
<tr>
<td>2: Semistarvation (n = 18)</td>
<td>6</td>
<td>16.9</td>
<td>0.6</td>
<td>&lt;10th</td>
<td>4.2</td>
<td>2.1</td>
<td>&lt;50th</td>
<td>9.1</td>
</tr>
<tr>
<td>3: Muscle atrophy (n = 31)</td>
<td>10</td>
<td>14.9</td>
<td>1.0</td>
<td>&lt;5th</td>
<td>7.2</td>
<td>1.4</td>
<td>&lt;90th</td>
<td>7.7</td>
</tr>
<tr>
<td>4: No impairment (n = 177)</td>
<td>56</td>
<td>18.4</td>
<td>1.7</td>
<td>&lt;50th</td>
<td>6.7</td>
<td>2.0</td>
<td>&lt;75th</td>
<td>9.6</td>
</tr>
<tr>
<td>Women</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1: Cachexia (n = 25)</td>
<td>27</td>
<td>13.5</td>
<td>0.9</td>
<td>&lt;5th</td>
<td>5.0</td>
<td>1.3</td>
<td>&lt;10th</td>
<td>6.1</td>
</tr>
<tr>
<td>2: Semistarvation (n = 5)</td>
<td>5</td>
<td>15.9</td>
<td>0.5</td>
<td>&lt;50th</td>
<td>4.7</td>
<td>1.1</td>
<td>&lt;5th</td>
<td>7.4</td>
</tr>
<tr>
<td>3: Muscle atrophy (n = 9)</td>
<td>10</td>
<td>14.5</td>
<td>0.7</td>
<td>&lt;10th</td>
<td>8.2</td>
<td>1.3</td>
<td>&lt;50th</td>
<td>6.0</td>
</tr>
<tr>
<td>4: No impairment (n = 55)</td>
<td>59</td>
<td>17.6</td>
<td>2.2</td>
<td>&lt;90th</td>
<td>9.6</td>
<td>3.5</td>
<td>&lt;75th</td>
<td>7.5</td>
</tr>
</tbody>
</table>

1 FFMI, fat-free mass index; FMI, fat mass index; SMI, skeletal muscle index. BMI was measured as kg/m². In men, cachexia is defined as a BMI <21 and an FMI <16; semistarvation is defined as a BMI <21 and an FMI ≥16; muscle atrophy is defined as a BMI ≥21 and an FMI <16; and no impairment is defined as a BMI ≥21 and an FMI ≥16. In women, cachexia is defined as a BMI <21 and an FMI <15; semistarvation is defined as a BMI <21 and an FMI ≥15; muscle atrophy is defined as a BMI ≥21 and an FMI <15; and no impairment is defined as a BMI ≥21 and an FMI ≥15. Because there was a significant difference between men and women in BMI (P < 0.05), we stratified mean values for FFMI, FMI, and SMI of the 4 body-composition categories by sex in Table 2 and related them separately to the sex-specific reference values reported by Schutz et al (18) and Janssen et al (19). The proportions of men to women did not differ significantly between the 4 body-composition categories. In categories 1 and 3, mean FFMI was below the 10th or even the 5th percentile in both male and female patients. Mean FFMI in categories 2 and 4 was within median range (<50th percentile) except for male patients in category 2, whose values were below the 10th percentile. FMI did not differ significantly between categories 1 and 2 or between categories 3 and 4 in either men or women. Although female patients with a low BMI had an FMI below the 10th percentile, male patients had an FMI within the median range. This indicates a sex-specific shift in body composition. When we applied the criteria for SMI and related physical disability that Janssen et al (19) used, a high proportion of the total patient population was characterized as being at moderate (51%) or high (31%) risk. In line with that study, male patients in the current study were at higher risk of physical disability than were female patients. On univariate Cox regression analysis, however, the association between SMI and survival in the current study did not differ significantly between the men (RR: 0.75; 95% CI: 0.65, 0.86) and the women (RR: 0.75; 95% CI: 0.51, 0.99). Furthermore, classification of the patients in the 4 categories clearly showed that, independent of sex, significantly (P < 0.001) more patients were at moderate to high risk of disability in the cachexia and muscle atrophy categories (ie, 1 and 3) than in semistarvation and no-impairment categories (ie, 2 and 4) (Table 2).

RESULTS

Patient characteristics are shown in Table 1. The mean age of the study group was 64 ± 9 y, 77% were male, and mean FEV1 was 36 ± 14% of predicted (range: 19–70%). Because there was a significant difference between men and women in BMI, FFMI, and FMI (all: P < 0.05), we stratified mean values for FFMI, FMI, and SMI of the 4 body-composition categories by sex in Table 2 and related them separately to the sex-specific reference values reported by Schutz et al (18) and Janssen et al (19). The proportions of men to women did not differ significantly between the 4 body-composition categories. In categories 1 and 3, mean FFMI was below the 10th or even the 5th percentile in both male and female patients. Mean FFMI in categories 2 and 4 was within median range (<50th percentile) except for male patients in category 2, whose values were below the 10th percentile. FMI did not differ significantly between categories 1 and 2 or between categories 3 and 4 in either men or women. Although female patients with a low BMI had an FMI below the 10th percentile, male patients had an FMI within the median range. This indicates a sex-specific shift in body composition. When we applied the criteria for SMI and related physical disability that Janssen et al (19) used, a high proportion of the total patient population was characterized as being at moderate (51%) or high (31%) risk. In line with that study, male patients in the current study were at higher risk of physical disability than were female patients. On univariate Cox regression analysis, however, the association between SMI and survival in the current study did not differ significantly between the men (RR: 0.75; 95% CI: 0.65, 0.86) and the women (RR: 0.75; 95% CI: 0.51, 0.99). Furthermore, classification of the patients in the 4 categories clearly showed that, independent of sex, significantly (P < 0.001) more patients were at moderate to high risk of disability in the cachexia and muscle atrophy categories (ie, 1 and 3) than in semistarvation and no-impairment categories (ie, 2 and 4) (Table 2).

In Figure 1, body-composition categories are linked to GOLD stage of disease severity. Most of the patients had advanced COPD (GOLD II, n = 71; GOLD III, n = 134; and GOLD IV,
n = 207). Cachexia was significantly (P = 0.001) more prevalent in GOLD stage IV than in GOLD stages II and III.

The mean duration of follow-up was 48 ± 20 mo, during which time 46% of the patients died. Univariate analysis showed that low BMI, FFMI, and FMI were significantly associated with increased mortality risk (Table 3). In addition, Cox stepwise regression analysis showed that FFMI was selected when BMI was no longer significant (data not shown) when entered as either an absolute value or as dichotomized BMI (< or ≥21); this indicates that FFMI is a stronger predictor for mortality than is BMI. After multivariate analysis, adjusted only for age (P = 0.001) and sex (NS) (model 1) or also adjusted for FEV1 (either in vol/s (data not shown) or as a percentage of predicted (P = 0.037)), IVC (NS), and resting arterial blood gases (NS) (model 2), FFMI was an independent predictor of mortality (P = 0.001 and 0.003 for model 1 and model 2, respectively), but FMI was not (Table 3). Although only a few patients were in the semistarvation category, the Kaplan-Meier plots for all the body-composition categories are shown in Figure 2. Survival was significantly (P < 0.001) less in patients with cachexia (26 mo; 95% CI: 21, 31 mo) and muscle atrophy (24 mo; 15, 33 mo) than in patients with semistarvation (36 mo; 28, 44 mo) or no impairment (47 mo; 37, 57 mo). The survival plot of the semistarvation category did not differ significantly from that of the no-impairment category during the first 3 y.

TABLE 3
Univariate and multivariate analysis of predictors of mortality

<table>
<thead>
<tr>
<th>Covariate</th>
<th>RR</th>
<th>95% CI</th>
<th>P</th>
<th>RR</th>
<th>95% CI</th>
<th>P</th>
<th>RR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.04</td>
<td>1.02, 1.06</td>
<td>0.001</td>
<td>1.04</td>
<td>1.02, 1.06</td>
<td>0.001</td>
<td>1.04</td>
<td>1.02, 1.06</td>
<td>0.001</td>
</tr>
<tr>
<td>Sex</td>
<td>0.72</td>
<td>0.50, 1.03</td>
<td>0.071</td>
<td>0.72</td>
<td>0.49, 1.06</td>
<td>0.010</td>
<td>0.84</td>
<td>0.56, 1.25</td>
<td>0.385</td>
</tr>
<tr>
<td>BMI</td>
<td>0.94</td>
<td>0.90, 0.97</td>
<td>0.001</td>
<td></td>
<td>—</td>
<td>—</td>
<td></td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>FFMI</td>
<td>0.88</td>
<td>0.83, 0.94</td>
<td>0.001</td>
<td>0.88</td>
<td>0.82, 0.94</td>
<td>0.001</td>
<td>0.90</td>
<td>0.84, 0.96</td>
<td>0.003</td>
</tr>
<tr>
<td>FMI</td>
<td>0.93</td>
<td>0.88, 0.99</td>
<td>0.009</td>
<td>1.01</td>
<td>0.94, 1.08</td>
<td>0.843</td>
<td>1.02</td>
<td>0.95, 1.09</td>
<td>0.680</td>
</tr>
<tr>
<td>FEV1</td>
<td>0.98</td>
<td>0.97, 0.99</td>
<td>0.001</td>
<td></td>
<td>—</td>
<td>—</td>
<td></td>
<td>0.98</td>
<td>0.97, 0.99</td>
</tr>
<tr>
<td>IVC</td>
<td>0.99</td>
<td>0.98, 0.99</td>
<td>0.016</td>
<td></td>
<td>—</td>
<td>—</td>
<td></td>
<td>0.99</td>
<td>0.99, 1.01</td>
</tr>
<tr>
<td>PaCO2</td>
<td>1.17</td>
<td>1.01, 1.38</td>
<td>0.047</td>
<td></td>
<td>—</td>
<td>—</td>
<td></td>
<td>0.98</td>
<td>0.81, 1.20</td>
</tr>
<tr>
<td>PaO2</td>
<td>0.84</td>
<td>0.77, 0.92</td>
<td>0.001</td>
<td></td>
<td>—</td>
<td>—</td>
<td></td>
<td>0.92</td>
<td>0.82, 1.02</td>
</tr>
</tbody>
</table>

1 RR, relative risk; FFMI, fat-free mass index; FMI, fat mass index; FEV1, forced expiratory volume in 1 s; IVC, inspiratory vital capacity; PaCO2, arterial carbon dioxide tension; PaO2, arterial oxygen tension.
2 FFMI, FMI, age, and sex (0 = women, 1 = men).
3 FFMI, FMI, age, sex (0 = women, 1 = men), FEV1, IVC, PaCO2, and PaO2.
4 Variable not included in the model.
category diverged from that of the no-impairment category to resemble the survival curves of the cachexia and muscle atrophy categories.

The Cox regression plots for the 4 categories, adjusted for age, sex, FMI, and disease severity markers, are shown in Figure 3. The RRs (95% CIs) of patients in the 3 tissue-depletion categories compared with those of patients in the no-impairment category were 1.91 (1.37, 2.67; P = 0.006), 1.23 (1.68, 2.24; NS), 1.96 (1.21, 3.17; P < 0.001) for categories 1 (cachexia), 2 (semistarvation), and 3 (muscle atrophy), respectively. Therefore, patients in both categories with low FFMI have a greater mortality risk than do patients with no impairment, and, thus, low BMI does not increase mortality risk beyond the increase due to low FFMI.

**DISCUSSION**

This study shows that FFMI is an independent predictor of mortality in COPD irrespective of FM and provides further support for the use of body-composition assessment as a systemic marker of disease severity in COPD staging. It also confirms a previous study of COPD that showed an independent association between lower-limb cross-sectional area and mortality, particularly in patients with FEV1 <50% (10). In the current study, however, BIA was used, which is an easier method of estimating FFMI in these patients without needing expensive apparatus or highly skilled technicians. Recently, Janssen et al (12) developed and crossvalidated BIA equations against magnetic resonance imaging in a sample of 388 men and women who varied widely in age and BMI. In that study, the correlation between BIA- and magnetic resonance imaging–measured muscle mass was 0.86. Gosker et al (20) showed in patients with COPD that FFMI assessed by BIA was significantly related to muscle fiber cross-sectional area taken from a biopsy of muscle tissue from the vastus lateralis, which indicated that whole-body FFMI also reflects lower-limb muscle atrophy in chronic disease. FFMI estimation via BIA was also used in a recent analysis of NHANES III that identified skeletal muscle cutoffs associated with a high likelihood of physical disability (19). With the use of these criteria, the current study group was characterized as being at moderate to high risk of physical disability. These studies all indicate that BIA may be a useful clinical screening instrument for characterization of the tissue-depletion pattern in chronic lung disease.

The findings of this study differ from those of population studies showing that an abundance of FM is particularly associated with increased cardiovascular disease–related mortality risk in overweight to obese subjects (reviewed in 21). The current study indicates that, in COPD patients, the association between FFMI and survival is independent of the amount of FM and that FFMI provides information to assist prognosis beyond that provided by BMI. Strikingly similar physical disability and mortality risks were seen in the cachexia and muscle atrophy categories, which indicated that a critical loss of muscle mass, not reflected in BMI, may be responsible for physical disability and greater mortality risk. In previous studies, we also showed significant differences in functional impairment such as exercise capacity measured by a 12-min walking test (7) or incremental cycle ergometry between patients in the cachexia and muscle atrophy categories relative to those in the semistarvation and no-impairment categories. Remarkably, the survival plot of the semistarvation category did not differ significantly from that of the no-impairment category during the first 3 y, whereas, thereafter, mortality was clearly higher in the semistarvation category. One could speculate that these patients initially have less mortality risk because of (relative) preservation of FFMI but that, in due course, they are at greater risk of a critical loss of FFMI. This hypothesis, however, should be confirmed in a longitudinal study using repeated measurements of body composition. The current study also showed remarkable sex-specific differences in body composition and disability risk from the reference values, and those differences warrant further investigation. As compared with the data of Schutz et al (18), the shift in body composition toward less FFMI and more FM was more striking in men than in women. One could argue that the male patients in the semistarvation category were not truly starving according to the external standards of Schutz et al (18). However, those patients clearly had lower FM than did patients in categories 3 and 4. Furthermore, in both the men and the women, comparison with the reference values of Janssen et al (19) showed that physical disability risk was clearly lower in categories 3 and 4, which confirmed previous studies relating those tissue-depletion categories to objective measures of skeletal function and exercise capacity (6–8). As was also indicated by Janssen et al (19), the SMI cutoffs were a significantly stronger predictor of physical disability in men than in women. This observation could indicate that SMI cutoffs should be adjusted. However, it might also be possible that the association between FFMI and physical disability risk, as judged by a questionnaire, really differs between males and females. This could reflect a different relation of body composition and functional capacity between males and females toward the functional limitations consequent to their chronic disease.
The relative preservation of FM in COPD patients could result from inactivity as a consequence of the progressive disability due to the disease. It could also be a consequence of biological factors such as a derangement in (fat) oxidative metabolism, as indicated by impaired β-adrenoceptor–mediated lipolysis, which reduced fat mobilization (22). That, in turn, could lead to an increase in glucose turnover and protein utilization, as was reflected in an increased whole-body protein turnover (23). The abovementioned shift in body composition could therefore be an indication of either accelerated sarcopenia or an early phase of cachexia. Because cachexia was seen mostly in GOLD IV COPD patients (characterized not only by severe airflow obstruction but also by PaO2 <8.0 kPa), the loss of FM as well as of FFM might be related to specific effects of hypoxia on energy balance.

FFM was associated with mortality with and without adjustment for static lung volumes and resting arterial blood gases. These markers are traditionally used to define disease severity and are included in the recent GOLD stages, but that does not exclude the possibility that other lung function markers might affect FFMI or the relation between FFMI and mortality. In particular, adjustment for the degree of emphysema would have been interesting, because previous studies showed that FFMI correlated with the diffusing capacity for carbon monoxide as a hallmark of emphysema (6). Furthermore, weight loss and low FFMI were significantly more common in patients with emphysema, as assessed by high-resolution computed tomography scanning, than in patients with chronic bronchitis (24). The process of emphysema might induce weight loss or loss of FFMI, but weight loss or loss of FFMI also may induce emphysema. Postmortem studies of persons who died in the Warsaw Ghetto during World War II suggested that death due to starvation was associated with pulmonary emphysema (25). In line with this observation but using advanced techniques to assess emphysema, an elegant study recently showed the presence of emphysema-like changes in the lungs of chronically malnourished anorexia nervosa patients (5).

On the basis of numerous reports showing an association between BMI and mortality risk in COPD, Celli at al (26) recently proposed that clinical staging for COPD should be based not only on spirometry and resting arterial blood gases, eg, the recent GOLD criteria, but also on BMI, exercise capacity, and dyspnea score. The results of the current study indicate that FFMI could be an even better independent predictor of systemic disease severity than is BMI.

The current study was limited by a skewed distribution of the study population toward more males and the more severe GOLD stages III and IV. In addition, categories 2 and 3 (semistarvation and muscle atrophy) were relatively small. Longitudinal studies with repeated assessments of body composition are needed to increase our knowledge of the sequence of body-composition changes so that we can better target therapeutic interventions. In advanced stages of disease, low FFM has already been clearly identified as a primary determinant of perceived disability, handicap, and health care costs, and it is therefore currently considered an important target for therapy (7, 17, 19, 27). Several studies specifically aimed therapeutic interventions at the accretion of muscle mass in patients with advanced COPD to improve functional capacity (28–33). The current study indicates that therapeutic strategies should depend not on BMI but on body composition. Although the studies by our group (28, 29, 31, 34) indicated that current therapeutic modalities can increase FFMI, the extent to which improvement in FFMI per se is reflected in other relevant outcome measures, including mortality, and the minimal clinically effective increase in FFMI remain to be investigated.

AMS and EFM set up the study. With the aid of RB, AMS analyzed the data and wrote the manuscript. CAW performed all body composition measurements. All authors read, commented on, and contributed to the manuscript. None of the authors had any personal or financial conflict of interest.

REFERENCES
20. Gosker HR, Engelen MP, van Mameren H, et al. Muscle fiber type IIx
Patterns of bioelectrical impedance vector distribution by body mass index and age: implications for body-composition analysis1–3

Anja Bosy-Westphal, Sandra Danielzik, Ralf-Peter Dörhöfer, Antonio Piccoli, and Manfred J Müller

ABSTRACT
Background: Bioelectrical impedance analysis (BIA) gives resistance (R) and reactance (Xc). R and Xc normalized for body height (H) can be plotted as a bivariate vector (H²/Xc versus H²/R). Vector BIA is useful for studying the determinants of BIA results.

Objective: We investigated the effect of age on BIA results and its relevance to body-composition analysis in a large database of impedance vector distributions stratified by age, sex, and body mass index (BMI).

Design: Mean bivariate vector distribution patterns (95% confidence ellipses) were examined in a German population of 15 605 children and adolescents and 213 294 adults. Children and adolescents were divided into 3 age groups with up to 5 BMI categories. In adults, 5 BMI categories were stratified into 7 age groups.

Results: Mean impedance vectors were shorter in children than in adults. The vector distribution pattern was influenced by sex, BMI, and age, with shorter vectors in females than in males and longer vectors with increasing BMI. Consistent with a decrease in body cell mass with increasing age, there was a downward slope in the mean vector with age as a result of a decrease in the H²/Xc vector component. By contrast, there was no age-dependent increase in the H²/R vector component. In women of the same BMI at different ages, H²/R and percentage fat mass tended to decrease with age.

Conclusions: The lack of an age-dependent increase in the H²/R vector component renders conventional BIA unsuitable for an examination of the age-related increase in body fat mass. By contrast, the increase in the H²/Xc vector component with advancing age suggests the potential of BIA to depict the age-related decrease in body cell mass.


KEY WORDS Bioelectrical impedance analysis, resistance, reactance, body mass index, children, adolescents, adults, aging

INTRODUCTION
Vector bioelectrical impedance analysis (BIA, RXc-graph method) is a pattern analysis of direct impedance measurements (resistance, R, and reactance, Xc) plotted as a bivariate vector standardized by the subject’s height (ie, expressing R/H and Xc/H in Ω/m). This method was described as a useful tool for monitoring hydration status in renal (1–3) and critical care (4) patients, in the follow-up of obese subjects during weight loss (3), and in infants (5–7). In the clinical setting, the advantages of vector BIA are convincing: no algorithms for conversion of raw impedance data into body-composition compartments are required. Results are therefore not biased by the choice of regression equation, the accuracy of the criterion method, or the selection criteria of the reference population. Because vector distribution patterns differ between men and women and by race or ethnicity and are dependent on body mass index (BMI) and age, national reference distributions for R and Xc used for body-composition analysis have been stratified accordingly (8, 9).

Vector distribution patterns reveal the determinants of BIA results; ie, the sex- and BMI-dependency of BIA results indicate that equations used for the prediction of body composition from impedance measurements need to be validated separately by sex and BMI classes (9). The age-dependency of vector distribution has not yet been critically viewed with regard to its relevance to body-composition analysis.

The present work aimed to investigate vector distribution patterns according to sex, BMI, and age in a large database of white children and adolescents (n = 16 237) and adults (n = 214 294). We used a modified version of the RXc-graph with the vector components H²/R and H²/Xc to analyze the relevance of age-related changes in vector BIA components for the description of age-related changes in body composition.

SUBJECTS AND METHODS
Subjects were recruited from 524 Precon-centers (commercial weight-management facilities) and the Kiel Obesity Prevention Study (KOPS) in Germany. Informed consent was obtained from all volunteers before participation. In case of children, parents provided written informed consent. Data comprising 10 127 girls (± SD age: 11.5 ± 3.9 y; range: 6–17 y), 6110 boys (9.5 ± 3.2 y; 6–17 y), 183 982 women (42.5 ± 13.2 y; 18–102 y), and 30 750 men (44.6 ± 13.5 y; 18–100 y) were collected over a period of 14 y from June 1990 to August 2003. Data from 420 girls and 212 boys were omitted because the children’s BMI (in

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2 Supported by a grant from Precon, Darmstadt, Germany.
3 Reprints not available. Address correspondence to MJ Müller, Institut für Humanenernährung und Lebensmittelkunde, Christian-Albrechts-Universität zu Kiel, Düsterborker Weg 17, D-24105 Kiel, Germany. E-mail: mmueller@nutrfoodsc.uni-kiel.de.
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for study participation by children. Body weight was measured to

There were no further selection criteria.

consisted of 15 605 children and adolescents (age range: 6–17 y)

BMI is in kg/m². \( R \), resistance; \( X_c \), reactance; \( H^2/R \) and \( H^2/X_c \). ANOVA showed significant interactions between age and BMI categories for both vector components (\( H^2/R \) and \( H^2/X_c \)).

kg/m²) exceeded the highest age-dependent BMI category. Likewise, we excluded 499 women and 62 men because of underweight (BMI < 18.5) and 761 women and 119 men because of severe obesity (BMI > 50). Thus, the final study population consisted of 15 605 children and adolescents (age range: 6–17 y) and 213 294 adults (age range: 18–102 y) who were examined by a total of 530 trained observers. Training of the investigators followed the same manual. All subjects were white, nonpregnant and nonlactating, and healthy (defined as the absence of a clinical condition that could influence fluid balance, e.g., renal, endocrine, or myocardial disease, as ascertained by participant questionnaire). There were no further selection criteria.

Data were acquired between 0700 and 1200. In the case of adults, all subjects were instructed on the importance of fasting overnight before measurement. Fasting was not a precondition for study participation by children. Body weight was measured to the nearest 0.1 kg and standing height to the nearest 0.5 cm with the subject in underwear and without shoes. BMI (kg/m²) was calculated with weight (kg) and height (m) measurements. A single tetrapolar BIA measurement of resistance \( (R) \) and reactance \( (X_c) \) was taken at a fixed frequency of 50 kHz between the right wrist and ankle (standard placement of surface electrodes) with a body impedance analyzer (BIA 2000-S; Data Input, Frankfurt, Germany) while the subjects were in a supine position on a nonconductive surface. (See reference 10 for a detailed description of the measurement procedure.) Mean CVs for within-day and between-day intraindividual measurements by the same observer were <2% for \( R \) and <3.5% for \( X_c \) and <1.5% for \( R \) and <5% for \( X_c \), respectively. The study was approved by the Ethical Committee of the Christian-Albrechts-University of Kiel.

### TABLE 1

<table>
<thead>
<tr>
<th>BMI</th>
<th>n</th>
<th>Height</th>
<th>( m )</th>
<th>( R )</th>
<th>( X_c )</th>
<th>( H^2/R )</th>
<th>( H^2/X_c )</th>
<th>( r )</th>
</tr>
</thead>
<tbody>
<tr>
<td>18–19 y</td>
<td>1052</td>
<td>1.68 ± 0.06</td>
<td>624.4 ± 61.7</td>
<td>64.7 ± 8.6</td>
<td>45.71 ± 5.65</td>
<td>444.6 ± 69.4</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>20–29 y</td>
<td>8307</td>
<td>1.68 ± 0.06</td>
<td>616.0 ± 60.9</td>
<td>64.3 ± 8.7</td>
<td>45.71 ± 5.64</td>
<td>447.0 ± 71.8</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>30–39 y</td>
<td>10162</td>
<td>1.67 ± 0.06</td>
<td>606.1 ± 61.9</td>
<td>63.9 ± 8.5</td>
<td>46.71 ± 5.77</td>
<td>446.9 ± 70.5</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>40–49 y</td>
<td>6691</td>
<td>1.66 ± 0.06</td>
<td>601.0 ± 61.2</td>
<td>62.6 ± 8.6</td>
<td>46.21 ± 5.76</td>
<td>447.8 ± 72.8</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>50–59 y</td>
<td>3408</td>
<td>1.65 ± 0.06</td>
<td>600.4 ± 60.2</td>
<td>60.2 ± 8.5</td>
<td>45.87 ± 5.59</td>
<td>462.6 ± 76.1</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>60–69 y</td>
<td>1106</td>
<td>1.64 ± 0.06</td>
<td>602.0 ± 62.6</td>
<td>57.9 ± 9.3</td>
<td>45.35 ± 5.90</td>
<td>478.1 ± 85.4</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>&gt;70 y</td>
<td>276</td>
<td>1.62 ± 0.06</td>
<td>597.3 ± 65.7</td>
<td>53.4 ± 9.9</td>
<td>44.46 ± 6.50</td>
<td>508.1 ± 105.1</td>
<td>0.59</td>
<td></td>
</tr>
</tbody>
</table>

*BMIs in kg/m². \( R \), resistance; \( X_c \), reactance; \( H^2/R \) and \( H^2/X_c \). ANOVA showed significant interactions between age and BMI categories for both vector components (\( H^2/R \) and \( H^2/X_c \)).
<table>
<thead>
<tr>
<th>BMI</th>
<th>Height</th>
<th>$R$</th>
<th>$X_c$</th>
<th>$H^2/R$</th>
<th>$H^2/X_c$</th>
<th>$r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15–20</td>
<td>115</td>
<td>1.82 ± 0.07</td>
<td>511.3 ± 53.9</td>
<td>61.0 ± 8.4</td>
<td>65.64 ± 8.46</td>
<td>555.8 ± 95.1</td>
</tr>
<tr>
<td>20–25</td>
<td>614</td>
<td>1.82 ± 0.07</td>
<td>510.1 ± 54.8</td>
<td>61.4 ± 7.9</td>
<td>65.51 ± 9.04</td>
<td>547.7 ± 91.3</td>
</tr>
<tr>
<td>25–30</td>
<td>639</td>
<td>1.81 ± 0.08</td>
<td>513.6 ± 55.3</td>
<td>69.8 ± 7.7</td>
<td>64.74 ± 8.55</td>
<td>560.9 ± 100.1</td>
</tr>
<tr>
<td>30–35</td>
<td>464</td>
<td>1.78 ± 0.07</td>
<td>512.2 ± 53.5</td>
<td>57.8 ± 7.2</td>
<td>62.92 ± 8.13</td>
<td>561.1 ± 91.6</td>
</tr>
<tr>
<td>35–40</td>
<td>294</td>
<td>1.77 ± 0.07</td>
<td>507.1 ± 53.0</td>
<td>55.4 ± 7.9</td>
<td>62.54 ± 8.74</td>
<td>578.6 ± 103.4</td>
</tr>
<tr>
<td>40–45</td>
<td>218</td>
<td>1.76 ± 0.07</td>
<td>509.7 ± 50.5</td>
<td>51.4 ± 8.1</td>
<td>61.52 ± 7.40</td>
<td>621.6 ± 119.6</td>
</tr>
<tr>
<td>&gt;70</td>
<td>86</td>
<td>1.73 ± 0.07</td>
<td>524.3 ± 59.9</td>
<td>46.6 ± 7.8</td>
<td>58.13 ± 7.26</td>
<td>666.2 ± 132.8</td>
</tr>
</tbody>
</table>

**RESULTS**

All data are given as means ± SDs. Statistical analyses were performed using SPSS for WINDOWS 8.0 (SPSS Inc, Chicago, IL). Pearson’s correlation coefficients were calculated for relations between $H^2/R$ and $H^2/X_c$. A $P$ value < 0.05 was considered as statistically significant. Differences between age or BMI groups were analyzed by ANOVA with Bonferroni post hoc test. Interactions among age and BMI categories were also investigated by ANOVA.

Confidence ellipses were calculated by using BIVA software (A Piccoli, G Pastori, Department of Medical and Surgical Sciences, University of Padova, Padova, Italy, 2002; available by E-mail: apiccoli@unipd.it). The calculation of confidence limits for mean impedance vectors requires bivariate normal distributions of $H^2/R$ and $H^2/X_c$ and is explained in detail by Piccoli et al (1, 9). Confidence ellipses describe the area in which the mean sex-, BMI-, and age-specific two-dimensional vectors fall within a 95% probability (1). This implies that graphically nonoverlapping 95% confidence ellipses are significantly different from each other ($P < 0.05$; conterminous with a significant Hotelling’s $T^2$ test; 1).

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1 BMI is in kg/m$^2$. $R$: resistance; $X_c$: reactance; $H$: height; $r$: correlation coefficient between $H^2/R$ and $H^2/X_c$. ANOVA showed significant interactions between age and BMI categories for both vector components ($H^2/R$ and $H^2/X_c$).
were stratified into 7 age groups (18–19, 20–29, 30–39, 40–49, 50–59, 60–69, and >70 y). Shown in Tables 1–3 are all the parameters necessary for calculation of the respective confidence or tolerance ellipses in subgroups of women (Table 1), men (Table 2), and children and adolescents (Table 3). The size of the confidence ellipses was influenced by the variability of the vector components and the sample size (smaller ellipses from a greater number of subjects with a similar SD). The shape of both the tolerance and the confidence ellipses was determined by the coefficient of correlation between $H^2/R$ and $H^2/Xc$. ANOVA showed significant interactions between age and BMI categories for both vector components ($H^2/R$ and $H^2/Xc$).

### Table 3: Impedance vector components in 9707 girls and 5898 boys by BMI and age classes

<table>
<thead>
<tr>
<th>Age 6–9 y</th>
<th>n</th>
<th>Height</th>
<th>$R$</th>
<th>Xc</th>
<th>$H^2/R$</th>
<th>$H^2/Xc$</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Girls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI 9–13</td>
<td>72</td>
<td>1.20 ± 0.11</td>
<td>879.4 ± 94.9</td>
<td>78.4 ± 14.9</td>
<td>16.86 ± 4.61</td>
<td>191.5 ± 52.6</td>
<td>0.77</td>
</tr>
<tr>
<td>BMI &gt;13–15</td>
<td>853</td>
<td>1.19 ± 0.06</td>
<td>829.5 ± 74.5</td>
<td>74.8 ± 32.1</td>
<td>17.44 ± 2.80</td>
<td>197.2 ± 34.9</td>
<td>0.71</td>
</tr>
<tr>
<td>BMI &gt;15–17</td>
<td>1168</td>
<td>1.21 ± 0.08</td>
<td>777.9 ± 71.2</td>
<td>72.0 ± 12.0</td>
<td>19.19 ± 3.60</td>
<td>209.5 ± 40.3</td>
<td>0.65</td>
</tr>
<tr>
<td>BMI &gt;17–25</td>
<td>879</td>
<td>1.30 ± 0.14</td>
<td>714.1 ± 79.8</td>
<td>67.8 ± 9.6</td>
<td>24.72 ± 8.02</td>
<td>259.4 ± 75.6</td>
<td>0.91</td>
</tr>
<tr>
<td>Boys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI 9–13</td>
<td>39</td>
<td>1.19 ± 0.06</td>
<td>858.2 ± 97.2</td>
<td>72.0 ± 12.7</td>
<td>16.85 ± 2.51</td>
<td>204.1 ± 39.7</td>
<td>0.74</td>
</tr>
<tr>
<td>BMI &gt;13–15</td>
<td>860</td>
<td>1.20 ± 0.06</td>
<td>787.8 ± 63.0</td>
<td>69.6 ± 9.4</td>
<td>18.59 ± 2.68</td>
<td>214.4 ± 71.3</td>
<td>0.33</td>
</tr>
<tr>
<td>BMI &gt;15–17</td>
<td>1194</td>
<td>1.22 ± 0.07</td>
<td>734.5 ± 66.7</td>
<td>67.1 ± 9.4</td>
<td>20.62 ± 9.35</td>
<td>225.3 ± 44.5</td>
<td>0.27</td>
</tr>
<tr>
<td>BMI &gt;17–25</td>
<td>670</td>
<td>1.28 ± 0.11</td>
<td>676.8 ± 70.3</td>
<td>64.5 ± 8.8</td>
<td>25.04 ± 6.55</td>
<td>263.1 ± 62.1</td>
<td>0.84</td>
</tr>
<tr>
<td>Boys</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>BMI 11–15</td>
<td>190</td>
<td>1.41 ± 0.07</td>
<td>816.0 ± 78.1</td>
<td>74.4 ± 9.9</td>
<td>24.54 ± 3.57</td>
<td>272.7 ± 55.2</td>
<td>0.74</td>
</tr>
<tr>
<td>BMI &gt;15–20</td>
<td>1113</td>
<td>1.44 ± 0.09</td>
<td>733.4 ± 79.1</td>
<td>69.9 ± 9.8</td>
<td>28.86 ± 5.50</td>
<td>304.5 ± 61.5</td>
<td>0.77</td>
</tr>
<tr>
<td>BMI &gt;20–25</td>
<td>748</td>
<td>1.53 ± 0.11</td>
<td>653.0 ± 71.5</td>
<td>63.8 ± 8.5</td>
<td>36.64 ± 7.92</td>
<td>376.1 ± 80.7</td>
<td>0.84</td>
</tr>
<tr>
<td>BMI &gt;25–30</td>
<td>654</td>
<td>1.59 ± 0.09</td>
<td>586.3 ± 62.8</td>
<td>58.7 ± 7.4</td>
<td>43.92 ± 7.82</td>
<td>439.7 ± 80.4</td>
<td>0.80</td>
</tr>
<tr>
<td>BMI &gt;30–35</td>
<td>293</td>
<td>1.63 ± 0.08</td>
<td>539.8 ± 55.1</td>
<td>55.2 ± 7.8</td>
<td>49.75 ± 7.55</td>
<td>490.2 ± 86.6</td>
<td>0.73</td>
</tr>
<tr>
<td>Boys</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>BMI 11–15</td>
<td>186</td>
<td>1.42 ± 0.06</td>
<td>786.2 ± 72.0</td>
<td>72.1 ± 8.9</td>
<td>25.93 ± 3.84</td>
<td>285.1 ± 51.0</td>
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</tr>
<tr>
<td>BMI &gt;15–20</td>
<td>1125</td>
<td>1.44 ± 0.07</td>
<td>701.2 ± 68.0</td>
<td>67.3 ± 8.7</td>
<td>29.91 ± 4.86</td>
<td>313.9 ± 58.0</td>
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<tr>
<td>BMI &gt;20–25</td>
<td>484</td>
<td>1.51 ± 0.10</td>
<td>636.0 ± 74.2</td>
<td>62.5 ± 7.9</td>
<td>36.60 ± 8.91</td>
<td>372.2 ± 84.9</td>
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<tr>
<td>BMI &gt;25–30</td>
<td>379</td>
<td>1.57 ± 0.10</td>
<td>576.3 ± 62.0</td>
<td>57.9 ± 6.9</td>
<td>43.61 ± 8.74</td>
<td>433.9 ± 82.6</td>
<td>0.84</td>
</tr>
<tr>
<td>BMI &gt;30–35</td>
<td>165</td>
<td>1.63 ± 0.11</td>
<td>527.5 ± 60.8</td>
<td>53.0 ± 6.4</td>
<td>51.27 ± 10.93</td>
<td>508.3 ± 95.0</td>
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<tr>
<td>BMI 14–19</td>
<td>157</td>
<td>1.68 ± 0.06</td>
<td>697.8 ± 73.4</td>
<td>68.5 ± 10.3</td>
<td>40.78 ± 5.30</td>
<td>420.9 ± 75.3</td>
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<tr>
<td>BMI &gt;19–25</td>
<td>1138</td>
<td>1.67 ± 0.06</td>
<td>624.2 ± 65.9</td>
<td>64.7 ± 8.5</td>
<td>45.40 ± 5.79</td>
<td>441.3 ± 69.8</td>
<td>0.72</td>
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<tr>
<td>BMI &gt;25–30</td>
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<td>578.0 ± 57.8</td>
<td>61.3 ± 8.1</td>
<td>48.61 ± 6.15</td>
<td>462.1 ± 73.6</td>
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<tr>
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<td>541.8 ± 53.9</td>
<td>57.1 ± 7.7</td>
<td>52.10 ± 6.55</td>
<td>489.9 ± 79.5</td>
<td>0.73</td>
</tr>
<tr>
<td>BMI &gt;35–40</td>
<td>271</td>
<td>1.67 ± 0.07</td>
<td>504.5 ± 54.2</td>
<td>53.7 ± 7.7</td>
<td>59.70 ± 7.94</td>
<td>529.9 ± 92.2</td>
<td>0.75</td>
</tr>
<tr>
<td>Boys</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI 14–19</td>
<td>34</td>
<td>1.74 ± 0.12</td>
<td>627.4 ± 72.8</td>
<td>62.7 ± 8.1</td>
<td>49.31 ± 9.97</td>
<td>494.1 ± 100.1</td>
<td>0.85</td>
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<tr>
<td>BMI &gt;19–25</td>
<td>167</td>
<td>1.76 ± 0.09</td>
<td>530.6 ± 62.0</td>
<td>59.1 ± 8.5</td>
<td>59.31 ± 10.01</td>
<td>535.7 ± 100.7</td>
<td>0.73</td>
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<tr>
<td>BMI &gt;25–30</td>
<td>252</td>
<td>1.75 ± 0.10</td>
<td>513.8 ± 64.7</td>
<td>55.6 ± 6.8</td>
<td>60.82 ± 12.06</td>
<td>558.8 ± 96.1</td>
<td>0.75</td>
</tr>
<tr>
<td>BMI &gt;30–35</td>
<td>249</td>
<td>1.76 ± 0.10</td>
<td>497.0 ± 58.5</td>
<td>52.3 ± 6.9</td>
<td>66.02 ± 11.68</td>
<td>605.0 ± 99.2</td>
<td>0.74</td>
</tr>
<tr>
<td>BMI &gt;35–40</td>
<td>94</td>
<td>1.77 ± 0.09</td>
<td>443.6 ± 50.4</td>
<td>48.9 ± 6.4</td>
<td>72.16 ± 11.28</td>
<td>656.0 ± 107.3</td>
<td>0.55</td>
</tr>
</tbody>
</table>

*BMI is in kg/m², R, resistance; Xc, reactance; H, height; r, correlation coefficient between $H^2/R$ and $H^2/Xc$. ANOVA showed significant interactions between age and BMI categories for both vector components ($H^2/R$ and $H^2/Xc$).*
decrease in the $H^2/R$ component with age. Concomitantly, a prediction equation for body composition that relied on $H^2/R$, $R$, and weight (11) did not show an age-related increase but rather a slight decrease in percentage fat mass in these women (Figure 4B). By contrast, the $H^2/Xc$ component decreased with increasing BMI and age (Figure 4C), which resulted in a decrease in body cell mass (calculated by the manufacturer’s algorithm as total body water/0.732 × phase angle × constant) with age in each BMI group (Figure 4D).

**DISCUSSION**

We established a large database of impedance vector distributions that exceeds current databases with respect to numbers and BMI and the age ranges covered. We used the database to analyze age-related changes in the vector components $H^2/R$ and $H^2/Xc$ with respect to their effects on body-composition analysis. The main result of our study was the insensitivity of conventional BIA to age-related changes in fat mass.

**Applications for vector BIA**

The RXc graph is a probability chart that relates an individual vector according to the mean value of the reference population (9). Sex-, BMI-, and age-specific vector distribution patterns 1) contribute to a better understanding of the factors influencing impedance results, 2) may be used a healthy reference when individual patient data are analyzed, and 3) serve as a quality criterion for BIA results before the application of algorithms to calculate total body water or fat-free mass. The comparison with reference vector distributions allows one to discriminate between impedance values that 1) lie within the normal range (and may therefore reasonably be converted into total body water or fat mass) or 2) lie outside the respective reference range and thus must not be converted into total body water or fat mass. If an individual vector does not match with its sex-, BMI-, and age-specific reference distribution, this is explained by either a measurement error (eg, false placement of electrodes) or increased or decreased hydration fraction of the fat-free mass (eg, in case of edema or dehydration). Vector BIA thus can be used as a measure of quality control to prevent misinterpretation of impedance readings and thus erroneous predictions of body compartments. A comparison of an individual impedance measurement with a reference database is easily achieved by using tolerance ellipses of the RXc graph. Tolerance ellipses are bivariate percentiles indicating the probability that an individual measurement falls at
a given distance from the observed mean vector of the reference population obtained with the same type of impedance analyzer. Tolerance ellipses for the $R/H$ and $X_c/H$ vector components have been reported for sex-specific populations comprising age ranges of 20–69 y and BMI ranges of 19 to 30 (8, 9).

**Determinants of vector distribution pattern**

Vector length is influenced by the amount of total body water and thus fat-free mass per conductor length. Men and obese subjects had greater fat-free mass per body height along with shorter $R/H$ or longer $H^2/R$ vector components (Figures 1–3; 1–3, 8, 9). By contrast, vector direction (phase angle) is influenced by soft tissue cell mass, which is modified by age in healthy subjects.

Fluid overload and increasing fat mass both lead to progressive vector shortening of the classic $R/H$ and $X_c/H$ vector components (1–3) or vector elongation of our modified vector components $H^2/R$ and $H^2/X_c$. Discrimination between the 2 conditions is possible, however. With increasing fat mass, the $H^2/X_c$ vector component remains in a fixed sex-dependent relation to the $H^2/R$ component (which is reflected by a constant phase angle with increasing BMI from 25 to 50; Figure 1). By contrast, fluid overload in renal patients was accompanied not only by vector shortening (lowering of the $R/H$ vector component) but also by vector down-sloping (lowering of phase angle), which could both be reversed after hemodialysis (3). Thus, fluid shifts occur along the major axis of a tolerance ellipse, and the upper and lower poles of the 75% tolerance ellipse represent the biological thresholds for clinically relevant dehydration and fluid overload, respectively (1, 2).

**Age-dependency of BIA variables**

There is evidence that body composition can be accurately estimated with conventional BIA by using sex-specific regression equations validated against a 4-compartment model in the age range of 12–94 y and BMI range of 14–39 (11). However, the lack of increase in the $H^2/R$ component with increasing age at the same BMI (Figure 4A) might explain the insensitivity of $H^2/R$ to age-related changes in body composition (Figure 4B). The use of a prediction equation for body composition that included both $H^2/R$ and $X_c$ (12) also failed to show an increase in percentage fat mass with age at the same BMI (results not shown). With the use of dual-energy X-ray absorptiometry or a 4-compartment model, mean percentages of body fat mass in 60–79-y-old women and men were shown to be 3% and 5% higher than the percentages in 20–39-y-old subjects of the same BMI (13). The inclusion of age as an independent variable in the regression equation might

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**FIGURE 2.** Mean impedance vectors with 95% confidence ellipses from children and adolescents (9707 girls and 5898 boys) stratified by sex, age, and BMI groups [BMI groups (in kg/m²) for each age and sex group are shown on the figure]. The numbers of subjects in each BMI and age category are given in Table 3, $H$, subjects' height; $R$, resistance; $X_c$, reactance. Graphically nonoverlapping 95% confidence ellipses are significantly different from each other, $P < 0.05$ (conterminous with a significant Hotelling's $T^2$ test).
therefore be indispensable for a plausible result, i.e., an increasing percentage fat mass with age at the same BMI. By contrast, age-related changes in body cell mass might be predictable by conventional BIA by applying prediction equations that include $X_c$ (Figure 4C and D). BIA prediction equations need to be developed and validated in the population under study (14, 15).

Some authors suggest that application of BIA to elderly populations requires only uniform validation procedures in the actual study population but not age-specific equations (16).

Study limitations

The limitations of our study derive from the selection criteria of the sample (i.e., inquiring rather than examining participants’ health status). Thus, we cannot exclude disturbances in fluid balance due to mild cardiac or renal insufficiency. However, because of the large sample size, the low variation in results (SD of $H^2/R$ and $H^2/X_c$), and the 95% CI, the effect of this bias should be negligible. Additionally, our study lacks independent data on body-composition analysis (e.g., measuring total body water by deuterium dilution) to confirm that individual vector placements solely determined by electrical properties of tissue per human conductor length relate to body composition independently of body shape and geometry. Future studies will be needed to examine this issue. Although we collected a huge database from centers located in different regions of Germany, our data cannot be considered to be representative of the German population. Because weight reduction was the main reason for monitoring nutritional status in the Precon centers, our data on overweight and obese subjects exceed the average of age- and sex-specific German population references (17). However, the aim of our study was to examine the patterns of bioelectrical impedance vector distribution by BMI and age. Thus, we only required a reasonable number of cases in each BMI and age group. The lack of representativeness of our study population as a whole is not contrary to the use of our data as a reference for the sex-, age-, and BMI-dependent distributions of $H^2/R$ and $H^2/X_c$ because ug
divided our subjects into subgroups according to sex and narrow ranges of BMI and age and 2) each subgroup consisted of a reasonable number of cases.

AB-W and MJM were responsible for the study design; AB-W, SD, and R-PD were responsible for data collection; AB-W and AP were responsible for data analysis; AB-W, AP, and MJM were responsible for discussion of data; and AB-W and MJM were responsible for writing of the manuscript. None of the authors had a conflict of interest.

REFERENCES
7. Savino F, Credi F, Grasso G, Oggero R, Silvestro L. The biagram vector:


Effect of whey on blood glucose and insulin responses to composite breakfast and lunch meals in type 2 diabetic subjects

Anders H Frid, Mikael Nilsson, Jens Juul Holst, and Inger ME Björck

ABSTRACT
Background: Whey proteins have insulinotropic effects and reduce the postprandial glycemia in healthy subjects. The mechanism is not known, but insulinogetic amino acids and the incretin hormones seem to be involved.

Objective: The aim was to evaluate whether supplementation of meals with a high glycemic index (GI) with whey proteins may increase insulin secretion and improve blood glucose control in type 2 diabetic subjects.

Design: Fourteen diet-treated subjects with type 2 diabetes were served a high-GI breakfast (white bread) and subsequent high-GI lunch (mashed potatoes with meatballs). The breakfast and lunch meals were supplemented with whey on one day; whey was exchanged for lean ham and lactose on another day. Venous blood samples were drawn before and during 4 h after breakfast and 3 h after lunch for the measurement of blood glucose, serum insulin, glucosedependent insulinotropic polypeptide (GIP), and glucagon-like peptide 1 (GLP-1).

Results: The insulin responses were higher after both breakfast (31%) and lunch (57%) when whey was included in the meal than when whey was not included. After lunch, the blood glucose response was significantly reduced (−21%; 120 min area under the curve (AUC)) after whey ingestion. Postprandial GIP responses were higher after whey ingestion, whereas no differences were found in GLP-1 between the reference and test meals.

Conclusions: It can be concluded that the addition of whey to meals with rapidly digested and absorbed carbohydrates stimulates insulin release and reduces postprandial blood glucose excursion after a lunch meal consisting of mashed potatoes and meatballs in type 2 diabetic subjects. Am J Clin Nutr 2005;82:69–75.

KEY WORDS Milk, whey, type 2 diabetes, blood glucose, serum insulin, incretin hormones

INTRODUCTION

In recent years, the awareness of the insulinotropic effects of milk has been growing (1). It seems that milk proteins, in particular the whey fraction, have a stimulating effect on insulin secretion in healthy subjects (2).

The key mechanism is not known, but elevated concentrations of specific insulinogetic amino acids as well as bioactive peptides, either originally present in whey or formed during digestion, are possible. Also, the incretin hormones seem to be involved. Particularly, glucose-dependent insulinotropic polypeptide (GIP) has been reported to increase significantly in blood plasma after whey ingestion (2). In addition to GIP, glucagon-like peptide 1 (GLP-1) is known to have insulinotropic properties during normal plasma glucose concentrations (3).

Previously, skim milk was reported to have insulinotropic effects in untreated type 2 diabetic subjects (4). It is known that proteins vary with respect to their effect on glucose metabolism in type 2 diabetic subjects and may stimulate insulin release and attenuate blood glucose response (5, 6). Food proteins are also capable of stimulating insulin response in the absence of carbohydrates (7, 8), and coinjection of dietary protein and glucose may have synergistic effects on insulin response (7).

The potential health aspects of the insulinotropic effects of milk remain unclear. Hyperinsulinemia, mediated from hyperglycemia, seems to be a risk factor for diseases within the metabolic syndrome. DelPrato et al (9) showed that experimental induction of hyperinsulinemia experimentally over a 48–72-h period at normoglycemic conditions decreased insulin sensitivity in healthy subjects. In contrast, epidemiologic evidence suggests that overweight subjects with a high intake of milk and dairy products are at lower risk of developing diseases related to the insulin resistance syndrome (10).

Clinically, fasting blood glucose rather than postprandial responses has been regarded as important as an indicator of the metabolic control of diabetes. However, the postprandial response is increasingly being recognized as a highly relevant determinant of glycated hemoglobin (Hb A1c) (11). Several insulin secretagogues are available on the market such as sulfonylureas and glinides for medical treatment of type 2 diabetes (12). It could be hypothesized that the insulinotropic effect of whey might be used similarly to those pharmaceuticals for the purpose of facilitating normoglycemia in diabetic subjects.

The aim of the present study was to investigate whether the insulinotropic effect of milk proteins, which was previously...
observed in healthy subjects, could be detected in type 2 diabetic patients. More specifically, we hypothesized that the insulinotropic effect of whey when used as a supplement to a breakfast and a subsequent lunch meal would lower the postprandial blood glucose response when compared with matched meals with no whey added. In addition, the responses of serum insulin, GIP, and GLP-1 were measured.

SUBJECTS AND METHODS

Test meals

White wheat bread (WWB) was baked at a commercial bakery (Koch’s Bageri, Klippan, Sweden) according to the recipe described by Liljeberg and Björck (13). After baking, the loaves were frozen and stored until use. In the afternoon before each test day, the bread was placed at ambient temperature for thawing overnight. On the morning of the test day, the crust was removed and the bread was sliced in pieces to provide 4 slices per portion.

Whey powder was obtained from Arla Foods (Stockholm, Sweden). Instant potato powder (Basmos; Procordia Food, Esslöv, Sweden) and meatballs (ICA Handlarna, Solna, Sweden) were bought at a local market.

The study included 2 separate test days, in random order ≥1 wk apart, for each person. On both occasions breakfast and lunch 4 h later were served, with or without the addition of whey. The breakfast consisted of 102 g WWB (corresponding to 44.7 g available carbohydrates; Table 1) and 300 g water. For lunch 52.2 g instant potato powder stirred in 270 g boiling water and 50 g meatballs were served. Also, 300 g water was included in the lunch meal.

On one of the test days, 27.6 g whey powder was dissolved in the water for breakfast and lunch. On the other test day, whey was exchanged for 5.3 g lactose dissolved in water and 96 g lean ham. Both protein and lactose content in the reference meals were equal to the quantities in the test meals. The amount of liquid was the same in all meals.

Chemical analysis

Lactose content in the whey powder was determined by using β-galactosidase to hydrolyze lactose enzymatically into galactose and glucose as earlier described by Nilsson et al (2). Glucose oxidase and peroxidase reagent (Glox-Novum; Kabi-Diagnostica, Stockholm, Sweden) dissolved in 0.5 mol/L trisphosphate buffer pH 7 (5.6 g/100 mL) was used to analyze the liberated amount of glucose.

The protein contents in whey powder, ham, and bread were determined by Kjeldahl analysis (Kjeltec Auto 1030 Analyser; Tecator, Höganäs, Sweden). WWB and potato powder were analyzed for starch content according to Holm et al (14). The nutritional composition of each meal is shown in Table 1.

Study design

Fourteen diet-treated subjects with type 2 diabetes, 6 women and 8 men, aged 27–69 y, with a mean (±SD) body mass index (in kg/m²) of 26.2 ± 3.1 were included in the study. Hb A₁c ranged from 4.3% to 7.7% (±SEM: 5.4 ± 0.2%; upper normal limit: 5.3%), and the mean (±SEM) fasting plasma glucose was 6.3 ± 1.2 mmol/L. The patients were recruited by advertising in local newspapers. Of those who responded, the first 14 to fulfill the inclusion criteria were chosen for the study. Medical records were obtained from the patient’s health care provider. The diagnosis was based on ≥2 fasting plasma glucose or postprandial plasma glucose measurements. Fasting plasma glucose > 6.9 mmol/L or postprandial (or postglucose load) plasma glucose > 12.1 mmol/L and absence of ketonemia and autoantibodies were considered diagnostic for diabetes type 2. None of the subjects had any known problems with lactose malabsorption.

The participants in the study were told to eat a few slices of WWB as a late meal in the evenings (between 2100 and 2200) before each test day. The subjects reported to the laboratory at 0745. A peripheral venous catheter was inserted into an antecubital vein, and a fasting blood sample was drawn. The breakfast was served, and the subjects ate steadily over a 12-min period. Black coffee or tea (150 mL) was served immediately after the breakfast. Each subject chose either coffee or tea at the first occasion and was then confined to the same drink throughout the study. Blood samples were drawn before breakfast (time 0) and at 10, 20, 30, 40, 60, 120, 180, and 240 min after breakfast commenced. Immediately after the 240-min sample, the subjects started eating lunch. Consequently, the 240-min value after breakfast was identical with the time 0 sample for lunch. Blood samples were also drawn at 10, 20, 30, 40, 60, 120, and 180 min after lunch. The lunch meal was eaten steadily during 12 min, and 150 mL coffee or tea was served afterward.

All meals were well tolerated, and the subjects had no problem finishing the meal within the 12-min period. All subjects were aware that they could withdraw from the study at any time. The study was conducted by an independent research organization, and the Ethics Committee of the Faculty of Medicine at Lund University approved the study.

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<td>Nutrient composition of the test meal and the reference meal</td>
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<td>Reference meal</td>
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<tr>
<td>Breakfast</td>
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<tr>
<td>Bread</td>
</tr>
<tr>
<td>Ham</td>
</tr>
<tr>
<td>Lactose</td>
</tr>
<tr>
<td>Total</td>
</tr>
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<td>Lunch</td>
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<tr>
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</tr>
<tr>
<td>Meatballs</td>
</tr>
<tr>
<td>Ham</td>
</tr>
<tr>
<td>Lactose</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Whey meal</td>
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<tr>
<td>Breakfast</td>
</tr>
<tr>
<td>Bread</td>
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<tr>
<td>Whey</td>
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<tr>
<td>Total</td>
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<tr>
<td>Lunch</td>
</tr>
<tr>
<td>Mashed potatoes</td>
</tr>
<tr>
<td>Meatballs</td>
</tr>
<tr>
<td>Whey</td>
</tr>
<tr>
<td>Total</td>
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</table>

1 According to the manufacturer.
Blood analysis

At all time points, blood glucose was measured in blood drawn from tubes containing EDTA with the use of a B-Glucose Analyzer (Hemocue AB, Ängelholm, Sweden). Plasma GIP and from tubes containing EDTA with the use of a B-Glucose Analyzer (Hemocue AB, Ängelholm, Sweden). Plasma GIP and 125I human GIP (70 MBq/nmol). For measurement of plasma GLP-1 (16) we used standards of synthetic GLP-1 7–36 amide and antiserum code no. 89390, which is specific for the amidated C-terminus of GLP-1 and, therefore, does not react with GLP-1–containing peptides from the pancreas. The rate of secretion of GLP-1 is accurately reflected because the assay measures the sum of intact GLP-1 and the primary metabolite, GLP-1 19–36 amide, into which GLP-1 is rapidly converted (17). For both assays sensitivity was 70% ethanol (by vol, final concentration). The C-terminally directed antiserum R65, which cross-reacts fully with human GIP but not with the so-called GIP 8000, whose chemical nature and relation to GIP secretion is uncertain, was used for the GIP radioimmunoassay (15). For standard and tracers we used human GIP and 125I human GIP (70 MBq/nmol). For measurement of plasma GLP-1 (16) we used standards of synthetic GLP-1 7–36 amide and antiserum code no. 89390, which is specific for the amidated C-terminus of GLP-1 and, therefore, does not react with GLP-1–containing peptides from the pancreas. The rate of secretion of GLP-1 is accurately reflected because the assay measures the sum of intact GLP-1 and the primary metabolite, GLP-1 19–36 amide, into which GLP-1 is rapidly converted (17). For both assays sensitivity was <1 pmol/L, intraassay CV <6% at 20 pmol/L, and recovery of standard, added to plasma before extraction, =100% when corrected for losses inherent in the plasma extraction procedure.

Calculations and statistical methods

The incremental areas under the curve (AUCs) for glucose, insulin, GIP, and GLP-1 were calculated for each subject and each meal by using GRAPH PAD PRISM (version 3.02; GraphPad Software Inc, San Diego, CA). All AUCs below the baseline were excluded from the calculations. The AUCs were expressed as means ± SEMs.

Significant differences among the AUCs were assessed with a general linear model (analysis of variance) followed by Tukey’s multiple comparisons test (MINITAB, release 13.32; Minitab Inc, State College, PA). Differences resulting in P values < 0.05 were considered significant.

The differences between the products at different time points were analyzed by using a mixed model (PROC MIXED in SAS release 8.01; SAS Institute Inc, Cary, NC) with repeated measures and an autoregressive covariance structure. When significant interactions between treatment and time were found, Tukey’s multiple comparisons test was performed for each time point (MINITAB, release 13.32; Minitab Inc).

RESULTS

Breakfast meal

The fasting blood glucose and serum insulin concentrations did not differ significantly between the days of the reference and the whey breakfasts. The postprandial blood glucose concentrations are shown in Figure 1. The blood glucose response after breakfast was not significantly different after the whey meal than with the reference meal when evaluating AUCs (0–60 min, 0–120 min, and 0–180 min; Table 2).

TABLE 2

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Reference</th>
<th>Whey</th>
<th>Change*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–60 min</td>
<td>168 ± 10.2</td>
<td>152 ± 12.9</td>
<td>-9</td>
</tr>
<tr>
<td>0–120 min</td>
<td>382 ± 32.0</td>
<td>370 ± 42.8</td>
<td>-3</td>
</tr>
<tr>
<td>0–180 min</td>
<td>450 ± 54.2</td>
<td>449 ± 65.8</td>
<td>0</td>
</tr>
<tr>
<td>Insulin AUC (nmol · min/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–60 min</td>
<td>7.3 ± 1</td>
<td>12.3 ± 1.7</td>
<td>68</td>
</tr>
<tr>
<td>0–120 min</td>
<td>25.5 ± 3.7</td>
<td>33.5 ± 4.3</td>
<td>31</td>
</tr>
<tr>
<td>0–180 min</td>
<td>37.5 ± 5.7</td>
<td>44.3 ± 6.1</td>
<td>18</td>
</tr>
<tr>
<td>GIP AUC (pmol · min/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–60 min</td>
<td>3231 ± 592</td>
<td>4605 ± 771</td>
<td>43</td>
</tr>
<tr>
<td>0–120 min</td>
<td>7562 ± 1319</td>
<td>9802 ± 1549</td>
<td>30</td>
</tr>
<tr>
<td>0–180 min</td>
<td>9565 ± 1631</td>
<td>11464 ± 1746</td>
<td>20</td>
</tr>
<tr>
<td>GLP-1 AUC (pmol · min/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–60 min</td>
<td>1356 ± 335</td>
<td>1343 ± 136</td>
<td>-1</td>
</tr>
<tr>
<td>0–120 min</td>
<td>2520 ± 507</td>
<td>2598 ± 276</td>
<td>3</td>
</tr>
<tr>
<td>0–180 min</td>
<td>2845 ± 563</td>
<td>3088 ± 343</td>
<td>9</td>
</tr>
</tbody>
</table>

* n = 14.

^ Change in postprandial response as a percentage of the reference meal.

± SEM (all such values).

^ Significantly different from reference, P < 0.05 (ANOVA followed by Tukey’s test).

FIGURE 1. Mean (±SEM) incremental changes (Δ) in blood glucose in response to equal amounts of carbohydrate from a reference meal (■) and a test meal of whey ( ▲ ) served as breakfast (A) and lunch (B) in 14 diabetic subjects. At breakfast, no significant treatment effect (P = 0.975) or treatment × time interaction (P = 0.262) was found. After lunch, no significant treatment effect (P = 0.057) was found, but a significant treatment × time interaction (P = 0.022) was found. Values with different lowercase letters are significantly different, P < 0.05 (Tukey’s test).
The postprandial insulin concentrations are shown in Figure 2. The insulin AUCs corresponding to the whey breakfast were significantly higher than the reference meal in the intervals 0–60, 0–120, and 0–180 min ($P < 0.05$; Table 2).

The GIP concentrations after the reference and whey meals are illustrated in Figure 3. The postprandial GIP response to the whey meal elicited higher responses than did the reference meal ($P < 0.05$) when examining the AUCs for the intervals 0–60, 0–120, and 0–180 min (Table 2).

GLP-1 concentrations are summarized in Figure 4. No significant differences in AUC for GLP-1 were found between the reference and whey meals (Table 2).

**Lunch meal**

The differences in blood glucose and serum insulin concentrations immediately before lunch (ie, 240 min after breakfast) were not statistically significant between the 2 test days. Significantly lower postprandial blood glucose responses (AUCs 0–60, 0–120, and 0–180 min) were observed when whey was included in the lunch than with the reference meal ($P < 0.05$; Table 3). The concomitant serum insulin response was elevated significantly after the whey meal than after the reference meal ($P < 0.05$). In accordance with the results after breakfast, the GIP responses after lunch (AUCs 0–60, 0–120, and 0–180 min) were significantly higher after the whey meal ($P < 0.05$; Table 3). Contrary to GIP, no significant difference was observed between the GLP-1 responses after whey compared with the reference meal when examining the AUCs.

**DISCUSSION**

Milk is known to have an insulinotropic effect (1), and it was recently shown that this property most probably is related to the whey protein fraction of milk (2). The results from the present study are in agreement with those earlier findings in healthy subjects whereby whey was found to elicit significantly higher insulin concentrations than WWB. Thus, from the present study it is evident that whey exhibits insulinotropic effects also in diet-treated diabetic subjects.

After breakfast no significant difference was observed between the whey meal and the reference meal containing ham when examining blood glucose. However, the glycemia was significantly decreased after lunch, most probably related to the higher insulin response, when whey was included in the meal. The cause for the less-pronounced insulinotropic effect of whey after breakfast is not known. Although the insulin response tended to be higher after the breakfast meal supplemented with whey, the differences in insulin response between the meal with whey added and the reference meal was smaller after breakfast (AUC 0–120 min differing 31%) than after lunch (AUC 0–120 min differing 57%). The lesser insulinotropic effect of whey after breakfast, in combination with the fact that the insulin resistance may be higher in the morning after the overnight fast (18), may explain the inability of whey to reduce the blood glucose increment after breakfast. In addition, the amounts of carbohydrates were slightly lower in the lunch meals than in the breakfast meals.

![Figure 2](image2.png)

**Figure 2.** Mean (±SEM) incremental changes ($\Delta$) in serum insulin in response to equal amounts of carbohydrate from a reference meal (■) and a test meal of whey (▲) served as breakfast (A) and lunch (B) in 14 diabetic subjects. At breakfast, no significant treatment effect ($P = 0.144$) was found, but a significant treatment × time interaction ($P = 0.046$) was found. After lunch, a significant treatment effect ($P = 0.011$) and treatment × time interaction ($P = 0.005$) were found at a given time. Values with different lowercase letters are significantly different, $P < 0.05$ (Tukey’s test).

![Figure 3](image3.png)

**Figure 3.** Mean (±SEM) incremental changes ($\Delta$) in glucose-dependent insulinotropic polypeptide (GIP) in response to equal amounts of carbohydrate from a reference meal (■) and a test meal of whey (▲) served as breakfast (A) and lunch (B) in 14 diabetic subjects. After breakfast, no significant treatment effect ($P = 0.072$) or treatment × time interaction ($P = 0.273$) was found. After lunch, no significant treatment ($P = 0.051$) or treatment × time interaction ($P = 0.307$) was found.
The key mechanism for the insulinotropic effects of milk proteins is not known. Certain amino acids may be involved (2), and a possible explanation for the differences in insulinotropic effects between various food proteins may be differences in their physical form. A liquid protein (whey) exits the stomach faster and is digested and absorbed more rapidly than a solid protein (19), resulting in a more pronounced postprandial plasma amino acid response.

Another possible pathway is through the activation of the incretin system. In parallel with insulin, the GIP concentrations were elevated in the blood shortly after ingestion when whey was included in the meal. This finding is in agreement with the earlier study in healthy subjects whereby whey was a much stronger GIP secretagogue than other food proteins such as cod, gluten, and cheese (2). The GIP response is possibly one key factor to the higher insulin response and the subsequent lowering of blood glucose seen after whey ingestion, at least in healthy subjects. In patients with type 2 diabetes, the insulinotropic effect of GIP is more uncertain because the incretin effect appears to be impaired as a consequence of deteriorated secretion of GLP-1 and loss of insulinotropic activity of GIP (20). Likely, the defective response to GIP may depend on the metabolic disturbances of diabetes (21).

GIP response is known to be mediated by carbohydrate and fat ingestion (22), whereas the effect of dietary protein is more uncertain, although stimulating effects have been reported (23, 24). In contrast, Nordt et al (25) registered no effect of GIP response in type 2 diabetic subjects after a protein-rich meal. Earlier, whey was found to stimulate the GIP and GLP-1 response compared with casein in healthy subjects but without a subsequent effect on insulin response (26).

Although the glucose-induced insulin secretion is impaired in type 2 diabetic patients, the insulin secretion of other nutrients may remain unaffected (27). Van Loon et al (27) reported a substantially higher insulin response (189%) when an amino acid and protein mixture (wheat protein) was coinjected with carbohydrates in type 2 diabetic patients but failed to show an attenuated glucose response. The insulinotropic effect of milk proteins, in particular the whey proteins, could be a valuable tool in the management of type 2 diabetes. Today sulfonylurea agents are commonly used to stimulate insulin secretion and to attenuate postprandial blood glucose. Occasionally, such treatment may cause hypoglycemia. This does not seem to be the case with GIP and GLP-1, and there have been no reports of whey causing hypoglycemia. Nuttall and Gannon (28) claimed that ingested protein in general is an efficient insulin secretagogue in type 2 diabetic subjects. It has been proposed that increasing insulin secretion may lead to early β-cell failure, the so-called “β-cell exhaustion hypothesis.” The validity of the hypothesis has, however, been hard to prove. One early landmark study from 1980 showed that treatment with sulfonylurea in patients with impaired glucose tolerance significantly decreased the clinical progression to overt diabetes mellitus rather than the contrary (29). In the United Kingdom Prospective Diabetes Study (30), patients with newly diagnosed type 2 diabetes were randomly assigned to either intensive or conventional treatment. It was found that the patients treated with insulin had the same rate of decline in HbA1c and fasting blood glucose than did patients treated with sulfonylurea. Consequently, the results from the United Kingdom Prospective Diabetes Study do not support the β-cell exhaustion hypothesis.

### TABLE 3

Postprandial areas under the curve (AUCs) for blood glucose, serum insulin, glucose-dependent insulinotropic polypeptide (GIP), and glucagon-like peptide 1 (GLP-1) after the reference and whey lunches, in diet-treated type 2 diabetic subjects.

<table>
<thead>
<tr>
<th></th>
<th>Reference</th>
<th>Whey</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose AUC (mmol - min/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–60 min</td>
<td>155 ± 12.3</td>
<td>124 ± 9.5</td>
<td>-20</td>
</tr>
<tr>
<td>0–120 min</td>
<td>353 ± 25.6</td>
<td>277 ± 26.8</td>
<td>-21</td>
</tr>
<tr>
<td>0–180 min</td>
<td>403 ± 35.0</td>
<td>320 ± 35.5</td>
<td>-21</td>
</tr>
<tr>
<td>Insulin AUC (nmol - min/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–60 min</td>
<td>7.3 ± 1.2</td>
<td>11.2 ± 1.1</td>
<td>53</td>
</tr>
<tr>
<td>0–120 min</td>
<td>17.0 ± 2.7</td>
<td>26.7 ± 3.1</td>
<td>57</td>
</tr>
<tr>
<td>0–180 min</td>
<td>21.5 ± 3.3</td>
<td>32.1 ± 4.2</td>
<td>49</td>
</tr>
<tr>
<td>GIP AUC (pmol - min/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–60 min</td>
<td>4323 ± 872</td>
<td>5872 ± 1041</td>
<td>36</td>
</tr>
<tr>
<td>0–120 min</td>
<td>9052 ± 1735</td>
<td>12658 ± 2442</td>
<td>40</td>
</tr>
<tr>
<td>0–180 min</td>
<td>11692 ± 2239</td>
<td>15656 ± 2889</td>
<td>34</td>
</tr>
<tr>
<td>GLP-1 AUC (pmol - min/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–60 min</td>
<td>1752 ± 230</td>
<td>1461 ± 197</td>
<td>-17</td>
</tr>
<tr>
<td>0–120 min</td>
<td>3404 ± 429</td>
<td>2907 ± 425</td>
<td>-14</td>
</tr>
<tr>
<td>0–180 min</td>
<td>4162 ± 511</td>
<td>3687 ± 593</td>
<td>-11</td>
</tr>
</tbody>
</table>

1 \( n = 14 \).
2 Change in postprandial response as a percentage of the reference meal.
3 ± SEM (all such values).
4 Significantly different from reference, \( P < 0.05 \) (ANOVA followed by Tukey’s test).
The present work is an acute study, and further studies are needed to determine possible long-term effects of whey on blood glucose control. However, recent data suggest that dietary protein might be useful to facilitate blood glucose control in subjects with type 2 diabetes by lowering both the 24-h glucose response and Hb A1c (31). Further studies are also needed to address the long-term metabolic effects of protein-induced hyperinsulinemia. Hoppe et al (32) recently showed that 1 wk with high milk intake increased insulin resistance and fasting serum insulin concentration. In contrast, epidemiologic data suggest that overweight subjects with a high intake of dairy products are at a lower risk of developing diseases related to the insulin resistance syndrome (10).

It has been proposed that reducing postprandial glycemia is a more expedient approach in diabetes treatment than lowering fasting blood glucose (11, 33). Conversely, others claim that in diabetic patients with fasting glucose > 7.8 mmol/L, the postprandial glucose response plays a much smaller role in determining the overall glycemic control (34). However, in both type 1 (35) and type 2 (36, 37) diabetic patients, postprandial glycemia was a better predictor for Hb A1c concentrations compared with fasting blood glucose. Increasing the endogenous insulin response by ingestion of an insulinotropic protein, or amino acid mixture, might improve glucose homeostasis in type 2 diabetic patients and could possibly postpone the introduction of medical treatment.

In the present study, the 180-min AUC was decreased by 21% when whey was included in the lunch meal than in the reference meal containing ham. This decline was in the same range as reported by Gribble et al (38) who registered a reduction of the plasma glucose increment during a 180-min period by 1.1–1.9 mmol/L and an 18% reduction in total postprandial (180 min) glucose exposure after different doses of nateglinide, a novel rapid-acting nonsulfonylurea insulin secretagogue. Kitabchi et al (39) reported a reduction of 2-h blood glucose AUC response with 12–24% after a standardized meal (Sustacal), after sulfonylurea therapy (glipizide and glyburide) during 6–15 mo.

In conclusion, the insulinotropic effect of whey proteins may potentially attenuate the postprandial blood glucose excursions over the day. The ability to amplify insulin secretion by specifically tailored amino acid mixtures is under investigation, and this approach may have fewer adverse effects than the commonly used therapeutic agents.

AHF recruited the subjects, collected the blood samples, was responsible for the analysis of blood glucose, and was involved in the design of the study and evaluation of the data. MN was involved in the design of the study, the statistical analysis, and the evaluation and was responsible for the insulin analysis. JH was responsible for the incretin analysis and was involved in the evaluation. IMEB was responsible for securing the funding and was involved in the design and the evaluation. All authors contributed to the writing of the paper. None of the authors had a conflict of interest.

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Co-ingestion of a protein hydrolysate and amino acid mixture with carbohydrate improves plasma glucose disposal in patients with type 2 diabetes

Ralph JF Manders, Anton JM Wagenmakers, René Koopman, Antoine HG Zorenc, Paul PCA Menheere, Nicolaas C Schaper, Wim HM Saris, and Luc JC van Loon

ABSTRACT
Background: Although insulin secretion after carbohydrate ingestion is severely impaired in patients with type 2 diabetes, amino acid and protein co-ingestion can substantially increase plasma insulin responses.

Objective: We investigated insulin responses and the subsequent plasma glucose disposal rates after the ingestion of carbohydrate alone (CHO) or with a protein hydrolysate and amino acid mixture (CHO + PRO) in patients with a long-term diagnosis of type 2 diabetes.

Design: Ten type 2 diabetic patients (mean ± SEM age: 62 ± 2 y; body mass index (kg/m²): 27 ± 1) and 9 healthy control subjects (age: 58 ± 1 y; body mass index: 27 ± 1) participated in 2 trials in which the plasma insulin response was measured after the ingestion of 0.7 g carbohydrate · kg⁻¹ · h⁻¹ with or without 0.35 g · kg⁻¹ · h⁻¹ of a mixture that contained a protein hydrolysate, leucine, and phenylalanine. Continuous infusions with [6,6-²H₂]glucose were then given to investigate plasma glucose disposal.

Results: Plasma insulin responses were higher by 299 ± 64% and 132 ± 63% in the CHO + PRO trial than in the CHO trial in the diabetic patients and the matched control subjects, respectively (P < 0.001). The subsequent plasma glucose responses were reduced by 28 ± 6% and 33 ± 3% in the CHO + PRO trial than in the CHO trial in the diabetic patients and the matched control subjects, respectively (P < 0.001). The reduced plasma glucose response in the diabetic patients was attributed to a 13 ± 3% increase in glucose disposal (P < 0.01).


KEY WORDS Glucose disposal, protein hydrolysate, leucine, phenylalanine, metabolism, type 2 diabetes

INTRODUCTION

The stimulating effect of the combined intake of carbohydrate and protein on plasma insulin release was reported in the 1960s (1, 2) and has since been confirmed in healthy subjects (3) and in patients with type 2 diabetes (4–6). Furthermore, intravenous infusion of free amino acids was reported to increase insulin secretion (7–9). In agreement with these findings, various in vitro studies with incubated β cells have attributed strong insulinotropic properties to arginine, leucine, and phenylalanine (10–17). We have performed various in vivo studies in which we defined an optimal insulinotropic amino acid and protein mixture containing leucine, phenylalanine, and a protein hydrolysate that has repeatedly been shown to augment the insulin response by an additional 100% in healthy subjects (18, 19). Nutritional interventions that effectively stimulate endogenous insulin secretion could be of particular significance in patients with type 2 diabetes. An increase in endogenous insulin secretion could increase blood glucose disposal and thus improve glucose homeostasis. Moreover, preventing or reducing the postprandial rise in blood glucose concentration that follows carbohydrate intake could reduce the risk of developing diabetic and cardiovascular complications (20, 21). Furthermore, the combined administration of amino acids and protein with carbohydrate, which leads to a state of hyperinsulinemia and hyperaminoacidemia, may represent an effective strategy to inhibit protein synthesis (22, 23). This outcome would be of particular interest, because muscle protein breakdown rates are markedly elevated in uncontrolled diabetes (24).

In patients with a long-term diagnosis of type 2 diabetes, hyperglycemia is not accompanied by a compensatory hyperinsulinemia. As such, it is generally assumed that the capacity of the β cell to secrete insulin is severely impaired as the result of several defects (25). These defects, which are all indicative of a progressive insensitivity of the β cell to glucose, include a reduced early-insulin secretory response to oral glucose, a reduced ability of the β cell to compensate for the degree of insulin resistance, a decline in the glucose-sensing ability of the β cell, and a shift to the right in the dose-response curve relating glucose
and insulin secretion (26). All of these defects involve glucose-sensing and -signaling pathways in the β cell. Although insulin secretion in response to carbohydrate intake is impaired in patients with type 2 diabetes, we recently showed that co-ingestion of a protein and amino acid mixture can increase the plasma insulin response 2–3-fold (27). Although such nutritional interventions can effectively stimulate endogenous insulin secretion in patients with a long-term diagnosis of type 2 diabetes, the clinical significance of these interventions in regard to blood glucose homeostasis remains to be established.

In the present study, we investigated the insulinotropic properties of a combination of a mixture of protein hydrolysate, leucine, and phenylalanine with carbohydrate and the glucose disposal rate after its ingestion in patients with a long-term diagnosis of type 2 diabetes and in healthy, matched control subjects.

SUBJECTS AND METHODS

Subjects

Ten male patients with a long-term diagnosis of type 2 diabetes and 10 healthy, matched control subjects were selected to participate in the present study. The baseline characteristics of the subjects are shown in Table 1. Exclusion criteria were impaired renal or liver function, obesity [body mass index (in kg/m²) >35], cardiac disease, hypertension, diabetic complications, and exogenous insulin therapy. All except one of the type 2 diabetic patients (n = 9) were using oral antidiabetic agents (metformin alone or in combination with sulfonylureas). One control subject withdrew from the experiment for personal reasons. In the type 2 diabetic patients, any medication being used was withheld for 2 d before the screening process. The subjects were screened for glucose intolerance and type 2 diabetes by use of a standard oral-glucose-tolerance test according to the World Health Organization criteria of 1999 (28). All subjects were informed about the nature and the risks of the experimental procedures before their written informed consent was obtained. All clinical trials were approved by the local medical ethical committee.

Screening

Before selection into the study, all subjects were given an oral-glucose-tolerance test. The subjects arrived at the laboratory at 0800 by car or public transportation after having fasted overnight. A blood sample was collected from the fasting subjects, after which a bolus of 75 g glucose (dissolved in 250 mL water) was ingested (t = 0 min). After 120 min, a second blood sample was obtained. Plasma glucose concentrations were measured to determine glucose intolerance and type 2 diabetes according to the World Health Organization criteria of 1999 (28). In addition, basal fasting plasma glucose and insulin concentrations were used to assess whole-body insulin resistance with the homeostasis model assessment insulin resistance index (29), which was calculated as the product of basal fasting plasma glucose (mmol/L) and insulin (mU/L) concentrations divided by 22.5.

Medication, diet, and activity before testing

Medication that stimulates insulin production or secretion (sulfonylurea derivatives) was withheld for 2 d before each test to prevent confounding effects on amino acid–induced insulin secretion. The use of insulin sensitizers (metformin) was continued to support the benefits of increasing endogenous insulin secretion on glucose homeostasis. All subjects maintained normal dietary and physical activity patterns throughout the entire experimental period. In addition, the subjects refrained from heavy physical labor and exercise for ≥3 d before each trial and filled out a food-intake diary for 2 d before the first trial to keep their dietary intake as identical as possible before the second trial. The evening before each trial, the subjects received a standardized meal (43.80 kJ/kg body wt that consisted of 60% of energy as carbohydrate, 28% of energy as fat, and 12% of energy as protein).

Design

Each subject participated in 2 trials, separated by a 2-wk period, in which the plasma insulin response and subsequent plasma glucose disposal rate were measured after the ingestion of 2 different beverage compositions (CHO, carbohydrate only; or CHO + PRO, carbohydrate and a mixture that contained a protein hydrolysate and the free amino acids leucine and phenylalanine). The subjects were placed in a supine position and remained inactive for 3 h. Drinks were provided in a randomized order and a double-blind fashion. The beverages were flavored to make the taste comparable in both trials (see below).

Protocol

The subjects reported to the laboratory at 0800 after an overnight fast. A catheter (Baxter BV, Utrecht, Netherlands) was inserted into an antecubital vein for the isotope infusion. Another catheter was inserted into a dorsal vein on the contralateral hand and was placed in a hot-box (60 °C) for arterialized blood sampling. After 10 min, a blood sample was collected from the

| TABLE 1 Subject characteristics
<table>
<thead>
<tr>
<th>Control group (n = 9)</th>
<th>Type 2 diabetic group (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>58.2 ± 1.0</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>84.89 ± 2.86</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.76 ± 0.02</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.49 ± 1.07</td>
</tr>
<tr>
<td>Basal plasma glucose (mmol/L)</td>
<td>5.31 ± 0.12</td>
</tr>
<tr>
<td>Plasma glucoseOGTTI20 (mmol/L)²</td>
<td>4.98 ± 0.41</td>
</tr>
<tr>
<td>Basal plasma insulin (mU/L)²</td>
<td>6.44 ± 0.90</td>
</tr>
<tr>
<td>Hb A₁c (%)</td>
<td>5.10 ± 0.13</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.52 ± 0.22</td>
</tr>
<tr>
<td>Diagnosed with type 2 diabetes (%)</td>
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</tr>
<tr>
<td>Medication</td>
<td>NA</td>
</tr>
</tbody>
</table>

¹ All values are x ± SEM. OGTT, oral-glucose-tolerance test; Hb A₁c, glycated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance (29); NA, not applicable; SU, sulfonylureas.
² Significantly different from control group, P < 0.01 (t test comparing patient and control group).
³ Plasma glucose concentration 2 h after ingestion of 75 g glucose. ⁴ Significantly different from basal values, P < 0.01 (t test comparing pre- and post-OGTT values).
resting subjects ($t = 0$ min). After the administration of an intravenous bolus of 13.5 μmol [6,6-2H2]glucose/kg, a continuous infusion of 277 ± 3 nmol·kg⁻¹·min⁻¹ of [6,6-2H2]glucose was started via a calibrated IVAC 560 pump (IVAC Corp, San Diego, CA) and continued until $t = 180$ min. At $t = 0$ min, the subjects drank an initial bolus (2 mL/kg) of the test drink (CHO or CHO+PRO). Repeated boluses (2 mL/kg) were ingested every 15 min until $t = 165$ min. Blood samples were drawn every 15 min during the first hour and then every 30 min until $t = 180$ min for the measurement of plasma glucose, glucose enrichment, and insulin. In addition, proinsulin and C-peptide concentrations were measured in the blood samples that were collected at $t = 0$, 60, 120, and 180 min.

**Beverages**

The subjects received repeated boluses of 2 mL/kg to ensure a given dose of 0.7 g carbohydrate·kg⁻¹·h⁻¹ (50% as glucose and 50% as maltodextrin) with or without 0.35 g·kg⁻¹·h⁻¹ of a protein hydrolysate and amino acid mixture (50% as casein hydrolysate, 25% as free leucine, and 25% as free phenylalanine) every 15 min until $t = 165$ min. Blood glucose and maltodextrin were obtained from AVEBE (Veenandam, Netherlands), crystalline amino acids were from BUFA (Uitgeest, Netherlands), and the casein protein hydrolysate was prepared by DSM Food Specialties (Delft, Netherlands). The casein hydrolysate (Insuvital; DSM Food Specialties) was obtained by enzymatic hydrolysis of sodium caseinate with the use of a neutral protease and a prolyl-specific endoproteinase. Both drinks were uniformly flavored by the addition of 0.2 g sodium saccharinate, 1.8 g citric acid, and 5 g of a cream vanilla flavor (Quest International, Naarden, Netherlands) for each 1 L of beverage.

**Isotope tracer calculations**

The glucose tracer (99% enriched; Cambridge Isotope laboratories, Andover, MA) was first dissolved in 0.9% saline. The glucose tracer concentration in the infusates averaged 22 ± 0.4 mmol/L. The [6,6-2H2]glucose infusion rate averaged 277 ± 3 nmol·kg⁻¹·min⁻¹. Plasma glucose enrichments are expressed as tracer/tracee ratios. The rate of appearance (Ra) and rate of disappearance (Rd) of glucose were calculated with the use of the single-pool non–steady state Steele equations (30) adapted for stable-isotope studies as described elsewhere (31).

\[
Ra = \frac{F - V[(C_2 + C_1)/2][E_2 - E_1]/(t_2 - t_1)]}{[E_2 + E_1]/2} \quad (1)
\]

\[
Rd = Ra - V \cdot [(C_2 - C_1)/(t_2 - t_1)] \quad (2)
\]

where $F$ is the infusion rate (in μmol·kg⁻¹·min⁻¹); $V$ is the distribution volume for glucose (160 mL/kg); $C_1$ and $C_2$ are the glucose concentrations (in mmol/L) at time 1 ($t_1$) and 2 ($t_2$), respectively; and $E_1$ and $E_2$ are the plasma glucose enrichments (tracer/tracee ratios) at time 1 and 2, respectively.

**Blood sample analysis**

Blood (10 mL) was collected in EDTA-containing tubes and centrifuged at 1000 × g for 10 min at 4 °C. Aliquots of plasma were immediately frozen in liquid nitrogen and stored at −80 °C until analyzed. Glucose concentrations (Uni Kit III; Roche, Basel, Switzerland) were analyzed with the COBAS FARA semiautomatic analyzer (Roche). Plasma insulin, proinsulin, and C-peptide concentrations were assayed with a modified, solid-phase, 2-site fluoroimmunometric assay based on a direct sandwich technique (DELFIA method; Perkin Elmer, Turku, Finland). To measure the glycated hemoglobin content, a 3-mL blood sample was collected in EDTA-containing tubes and was analyzed by HPLC (Bio-Rad Diamat, Munich, Germany). After derivatization of the plasma samples, plasma [6,6-2H2]glucose enrichment was measured by electron ionization gas chromatography–mass spectrometry (Finnigan INCOS-XL; Finnigan Mat, Hemel, Hemstead, United Kingdom).

**Statistics**

Data are expressed as means ± SEMs. The plasma responses were calculated as the area under the curve minus baseline values. To compare plasma metabolite concentrations and tracer kinetics over time between trials, a two-way repeated-measures analysis of variance (ANOVA) was performed. Subgroups were analyzed further whenever significant time-by-treatment interactions were observed. Changes in time within each group were checked for statistical significance with the use of a one-way repeated-measures ANOVA. When an $F$ ratio was significant, a Scheffe’s post hoc test was performed to locate specific differences. For non-time-dependent variables, a multiway ANOVA alone or with a Student’s $t$ test for unequal observations was used. Significance was set at $P < 0.05$. All calculations were performed with STATVIEW 5.0 (SAS Institute Inc, Cary, NC).

**RESULTS**

**Insulin**

Plasma insulin concentrations in subjects that had fasted overnight were similar in both groups and in both trials. Insulin concentrations increased significantly in both groups after the ingestion of carbohydrate alone and carbohydrate with the protein and amino acid mixture ($P < 0.001$; Figure 1A). A repeated-measures ANOVA showed a significant time–by-treatment interaction for plasma insulin concentrations ($P < 0.01$). After $t = 60$ min, plasma insulin concentrations in the diabetes group were higher in the CHO+PRO trial than in the CHO trial ($P < 0.05$). No significant differences were found between trials in the control group. After the insulin response was expressed as the area under the curve (minus baseline values), significantly greater plasma insulin responses were observed in the CHO+PRO trial than in the CHO trial in both groups ($P < 0.01$, Figure 1B). Plasma insulin responses were 299 ± 64% and 132 ± 63% greater in the CHO+PRO trial than in the CHO trial in the diabetes and control groups, respectively ($P < 0.01$).

**C-peptide and proinsulin**

Plasma C-peptide concentrations in fasting subjects were similar in both groups. A repeated-measures ANOVA showed a significant time–by-treatment interaction for plasma C-peptide concentrations ($P < 0.01$). In both trials, C-peptide concentrations increased significantly over time ($P < 0.05$; Figure 2). After $t = 60$ min, plasma C-peptide concentrations in the diabetes group were significantly higher in the CHO+PRO trial than in the CHO trial ($P < 0.05$). When expressed as the area under the curve, significantly greater C-peptide responses were observed in the CHO+PRO trial than in the CHO trial in both groups ($P < 0.01$). Plasma C-peptide responses were 98 ± 18% and 56 ± 26%
greater in the CHO+PRO trial than in the CHO trial in the diabetes and control groups, respectively ($P < 0.01$). Plasma C-peptide concentrations correlated well with plasma insulin concentrations ($r = 0.89$, $P < 0.001$).

Plasma proinsulin concentrations in fasting subjects were higher in the type 2 diabetes group than in the normoglycemic control subjects ($28.3 \pm 2.9 \text{ mmol/L}$ compared with $7.5 \pm 0.5 \text{ mmol/L}$, respectively $P < 0.01$). In both trials, proinsulin concentrations increased significantly over time ($P < 0.01$; Figure 2) and showed a significant time-by-treatment interaction ($P < 0.01$). After $t = 120 \text{ min}$, plasma proinsulin concentrations in the diabetes group were higher in the CHO+PRO trial than in the CHO trial ($P < 0.05$). No significant differences were observed between trials in the control group. When expressed as the area under the curve, significantly greater proinsulin responses were observed in the CHO+PRO trial than in the CHO trial in both groups ($P < 0.05$). The plasma proinsulin responses were $151 \pm 28\%$ and $84 \pm 37\%$ greater in the CHO+PRO trial than in the CHO trial in the diabetes and the control groups, respectively ($P < 0.05$). Plasma proinsulin concentrations correlated with both plasma insulin and plasma C-peptide concentrations ($r = 0.79$ and $r = 0.85$, respectively; $P < 0.001$).
Glucose

Plasma glucose concentrations in fasting subjects were higher in the type 2 diabetic patients than in the normoglycemic control subjects (9.7 ± 0.3 mmol/L compared with 5.7 ± 0.1 mmol/L, respectively; P < 0.01). A repeated-measures ANOVA showed a significant time-by-treatment interaction for plasma glucose concentrations (P < 0.01). In the type 2 diabetic patients, plasma glucose concentrations in the CHO trial increased after carbohydrate ingestion until t = 150 min, after which values reached a plateau. In the CHO + PRO trial, glucose concentrations increased significantly during the first 90 min (P < 0.01), after which they either reached a plateau or tended to decline (Figure 3A). At t = 180 min, the plasma glucose concentration was significantly lower in the CHO + PRO trial than in the CHO trial (P < 0.05) for the type 2 diabetes group. In the control group, plasma glucose concentrations slightly increased during the first 60 min in both trials and then returned to baseline levels over the next 2 h (Figure 3A). Plasma glucose concentrations were significantly higher in the type 2 diabetic patients than in the matched control subjects (P < 0.05). After expressing the plasma glucose response as the area under the curve, we observed a significantly higher plasma glucose response in the type 2 diabetic patients than in the matched normoglycemic control subjects (P < 0.001; Figure 3B). In both groups, significantly lower plasma glucose responses were observed in the CHO + PRO trial than in the CHO trial (P < 0.001; Figure 3B). The plasma glucose response was 28 ± 6% and 33 ± 3% lower in the CHO + PRO trial than in the CHO trial in the diabetes and matched control groups, respectively (P < 0.001).

Glucose tracer kinetics

In the type 2 diabetes group, the plasma glucose Ra was stable over the entire testing period and averaged 42.4 ± 0.8 and 41.2 ± 1.1 μmol · kg⁻¹ · min⁻¹ in the CHO and CHO + PRO trials, respectively. In the control group, the plasma glucose Ra was also stable and averaged 39.8 ± 0.7 and 37.9 ± 0.8 μmol · kg⁻¹ · min⁻¹ in the CHO and CHO + PRO trials, respectively (Table 2 and Figure 4A and B). No significant differences in the plasma glucose Ra were observed between trials or groups.

The glucose Rd increased over time in both trials and in both groups (P < 0.05; Figure 4C and D). In the diabetes group, the Rd averaged 19.7 ± 2.4 and 20.4 ± 2.8 μmol · kg⁻¹ · min⁻¹ at t = 30 min and increased over time to reach 45.1 ± 1.8 and 45.4 ± 3.6 μmol · kg⁻¹ · min⁻¹ in the CHO and CHO + PRO trials, respectively (Figure 4C). In the control group, the Rd averaged 14.7 ± 1.4 and 19.4 ± 1.7 μmol · kg⁻¹ · min⁻¹ at t = 30 min and increased to 45.4 ± 2.4 and 44.8 ± 2.2 μmol · kg⁻¹ · min⁻¹ in the CHO and CHO + PRO trials, respectively (Figure 4D). The increase in the Rd over time was significantly different between groups (P < 0.05).

Plasma glucose disposal, expressed as the percentage of the appearing glucose that disappears from the circulation, was significantly lower in the diabetic patients than in the matched control subjects (P < 0.001; Table 2). In the diabetes group, plasma glucose disposal was 12.5 ± 3.1% higher in the CHO + PRO trial than in the CHO trial (P < 0.01). In the control group, plasma glucose disposal was not significantly improved in the CHO + PRO trial (3.4 ± 2.2%; P = 0.2; Table 2).

In the diabetes group, the glucose disposal rate was significantly improved by 15.8 g (≈88 mmol) over the 150-min period in the CHO + PRO trial compared with the CHO trial (P < 0.01). In the control group, an additional 11.7 g (≈65 mmol) glucose was disposed of during the 150-min period in the CHO + PRO trial compared with the CHO trial (P = 0.2).

DISCUSSION

The present study showed that co-ingestion of carbohydrate with a mixture containing casein hydrolysate, leucine, and phenylalanine substantially increased insulin secretion when compared with the ingestion of carbohydrate alone. The substantial
between groups): Control group (n = 9)  

<table>
<thead>
<tr>
<th></th>
<th>CHO</th>
<th>CHO + PRO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ra (µmol·kg⁻¹·min⁻¹)</td>
<td>39.8 ± 0.7</td>
<td>37.9 ± 0.8</td>
</tr>
<tr>
<td>Rd (µmol·kg⁻¹·min⁻¹)</td>
<td>36.2 ± 1.7</td>
<td>35.7 ± 1.3</td>
</tr>
<tr>
<td>Glucose disposal (Ra as % of Ra)</td>
<td>91 ± 4</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>Time for Rd to match Ra (min)</td>
<td>90 ± 8</td>
<td>75 ± 6²</td>
</tr>
</tbody>
</table>

Type 2 diabetic group (n = 10)  

<table>
<thead>
<tr>
<th></th>
<th>CHO</th>
<th>CHO + PRO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ra (µmol·kg⁻¹·min⁻¹)</td>
<td>42.4 ± 0.8</td>
<td>41.2 ± 1.1</td>
</tr>
<tr>
<td>Rd (µmol·kg⁻¹·min⁻¹)</td>
<td>30.3 ± 1.3</td>
<td>33.2 ± 1.5</td>
</tr>
<tr>
<td>Glucose disposal (Ra as % of Ra)</td>
<td>72 ± 3²</td>
<td>81 ± 3²</td>
</tr>
<tr>
<td>Time for Rd to match Ra (min)</td>
<td>179 ± 8³</td>
<td>135 ± 9⁴</td>
</tr>
</tbody>
</table>

All values are ± SEM of [6.6-² H][glucose tracer rate of appearance (Ra) and disappearance (Rd) and Rd expressed as percentage of Ra over the entire 150-min period. CHO, carbohydrate; CHO + PRO, carbohydrate and protein mixture.

² Significantly different from CHO trial, P < 0.01 (t test comparing trials within each group).
³,⁴ Significantly different from control group (t test comparing trials between groups); ²P < 0.001, ³P < 0.01.

3–4-fold greater insulin response significantly improved postprandial glucose disposal and resulted in lower plasma glucose concentrations in type 2 diabetic patients. This study indicates that nutritional interventions that improve endogenous insulin secretion can be practical and effective tools in the treatment of type 2 diabetes.

The synergistically stimulating effect of the combined ingestion of carbohydrate and intact protein on plasma insulin release was first reported in the late 1960s (1, 2) and was later confirmed in both healthy subjects (3) and type 2 diabetic patients (4–6). Floyd et al (7–9, 32) investigated the effects of intravenous infusions of various amino acids on plasma insulin secretion and reported that arginine, leucine, and phenylalanine were the most insulinotropic amino acids. We have confirmed many of these findings after testing the oral administration of these amino acids in combination with carbohydrate (18, 19). Consequently, we defined a practical and optimal insulinotropic amino acid and protein mixture composed of a protein hydrolysate, free leucine, and phenylalanine (18, 19). Recently, we investigated the insulinotropic properties of this mixture in patients with a long-term diagnosis of type 2 diabetes and reported a 189% greater plasma insulin response in these patients when the mixture was co-ingested with carbohydrate than when carbohydrate was ingested alone (27). Although that study clearly showed that endogenous insulin secretion can be substantially increased in patients with a long-term diagnosis of type 2 diabetes, the clinical relevance of these findings had not yet been established. Therefore, in the present study, we investigated plasma glucose disposal after the ingestion of carbohydrate with or without the addition of such an insulinotropic protein hydrolysate and amino acid mixture in healthy subjects and in type 2 diabetic patients.

The patients with type 2 diabetes who were selected for this study had been diagnosed with type 2 diabetes for ≥10 y. Basal fasting glucose concentrations, oral-glucose-tolerance test values, glycated hemoglobin content, and the homeostasis model assessment insulin resistance index values confirmed their type 2 diabetic state (Table 1). Hyperinsulinaemia, a compensatory response to the prevailing hyperglycaemia, was no longer present in these patients (Table 1 and Figure 1). After ingestion of only carbohydrate in the CHO trial, insulin responses were substantially lower in the diabetic patients than in the control subjects (Figure 1B). This finding clearly illustrates the reduced sensitivity of the β cell to glucose in the type 2 diabetic state (26). Interestingly, co-ingestion of carbohydrate with the protein hydrolysate and amino acid mixture in the CHO+PRO trial significantly increased the plasma insulin response by 299 ± 64% and 132 ± 63% in the diabetic patients and the normoglycemic control subjects, respectively (P < 0.01; Figure 1B). The insulin response in the CHO+PRO trial in the type 2 diabetic patients was similar to the insulin response reported in the CHO trial in the healthy subjects (Figure 1B). In other words, although the sensitivity of the pancreas to carbohydrate intake is significantly reduced in patients with a long-term diagnosis of type 2 diabetes, the capacity to secrete insulin in response to other stimuli (such as amino acids) remains intact. Therefore, the defects in the insulin response after the ingestion of a meal in these patients are mainly attributed to the reduced sensitivity of the β cell to glucose and not to an overall defect in the capacity to produce or to secrete insulin.

To confirm that the elevated plasma insulin concentrations in the CHO+PRO trial are indeed secondary to increased insulin production, we measured plasma C-peptide and proinsulin concentrations according to the method of Hovorka and Jones (33). In the process of insulin production, the precursor proinsulin is cleaved into insulin and the 31-kD residue connecting peptide (C-peptide). Insulin, C-peptide, and a small amount of residual proinsulin are stored in the secretory granules of the β cell until secretion (34). In the present study, we observed a significant increase in plasma C-peptide and proinsulin concentrations over time in all trials (Figure 2). Significantly greater plasma C-peptide responses were observed in the CHO+PRO than in the CHO trial (98 ± 18% and 56 ± 26% in the diabetic patients and healthy control subjects, respectively; P < 0.01). Similarly, plasma proinsulin responses were also 151 ± 28% and 84 ± 37% greater in the CHO+PRO trial than in the CHO trial in the diabetes and control groups, respectively (P < 0.05). Both C-peptide and proinsulin concentrations correlated well with plasma insulin concentrations (r = 0.89 and r = 0.79, respectively; P < 0.001). Thus, these data further support the observation that co-ingestion of carbohydrate with the protein and amino acid mixture in the CHO+PRO trial effectively stimulates de novo insulin production.

In response to the increased insulin production and secretion rate in the CHO+PRO trial, plasma glucose concentrations were significantly decreased when compared with values observed in the CHO trial (Figure 3A). In the CHO+PRO trial, plasma glucose responses were decreased by as much as 28 ± 6% and 33 ± 3% in the diabetic patients and normoglycemic control subjects, respectively, compared with responses in the CHO trial (P < 0.001). This decrease in the plasma glucose response is much more prominent than in our earlier observations (27), which can be explained by the longer trial duration in the present study. Interventions that effectively reduce the postprandial rise in plasma glucose concentrations after carbohydrate intake are of clinical significance and have been associated with a reduced risk of developing diabetic and cardiovascular complications (20, 21). Many food components or pharmacologic agents that effectively lower postprandial glucose concentration after meal ingestion inhibit gastric emptying or intestinal uptake of glucose or both (35–37). In the present study, we applied a continuous
infusion of a [6,6-2H2]glucose tracer to measure the Ra of glucose in the circulation. Plasma glucose Ras were similar in both groups and trials and remained constant throughout the trials (Table 2, Figure 4A and B). This finding indicates that inhibition of gastrointestinal uptake of glucose is not responsible for the observed decline in the postprandial blood glucose response after co-ingestion of carbohydrate with the protein and amino acid mixture.

Whereas the plasma glucose Ra remained stable throughout the trials, the plasma glucose Rd from the circulation significantly increased over time in both trials (Figure 4; \(P < 0.01\)). In contrast with the Ra values, the plasma glucose Rd was strikingly different between the diabetic patients and the healthy, matched control subjects (Figure 4C and D). Whereas Rd values increased exponentially in the control subjects, a more gradual rise in the glucose Rd was observed in the diabetic patients (\(P < 0.01\)). It took about twice as long in the diabetic patients as in the healthy subjects for the plasma glucose Ra to be matched by its Rd (\(P < 0.01\)). Consequently, plasma glucose disposal (calculated as Rd expressed as a percentage of Ra) was significantly lower in the diabetic patients than in the normoglycemic control subjects (Table 2; \(P < 0.01\)). Accordingly, plasma glucose disposal after co-ingestion of carbohydrate with the protein and amino acid mixture improved plasma glucose disposal by 13 ± 3% (\(P < 0.01\)) and 3 ± 2% (\(P = 0.2\)) in diabetic patients and healthy control subjects, respectively.

In conclusion, ingestion of a protein hydrolysate, leucine, and phenylalanine mixture can substantially augment insulin responses after carbohydrate intake. In patients with a long-term diagnosis of type 2 diabetes, co-ingestion of carbohydrate with such a mixture can induce a 3–4-fold greater plasma insulin response than ingestion of carbohydrate alone. This response effectively improves plasma glucose disposal and thereby reduces the postprandial plasma glucose concentration. The combined ingestion of an amino acid and protein mixture with carbohydrate represents an effective interventional strategy in the treatment of type 2 diabetes.

We thank Jos Stegen, Joan Senden, and Annemie Gijsen for their expert analytic assistance and thank the subjects who volunteered to participate in these trials for their enthusiastic support.

RJFM, AJMW, and LJCvL designed the study. RJFM organized and carried out the clinical trials with the assistance of RK and AHGZ. RJFM performed the statistical analysis and wrote the manuscript together with LJCvL. PPCAM performed the plasma proinsulin, insulin, and C-peptide
analyses, NCS and WHMS provided medical assistance. None of the authors had a personal or financial conflict of interest.

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Absorption of calcium from tortilla meals prepared from low-phytate maize

K Michael Hambidge, Nancy F Krebs, Jamie L Westcott, Lei Sian, Leland V Miller, Kevin L Peterson, and Victor Raboy

ABSTRACT
Background: Calcium fortification of maize has been achieved for millennia in Central America by the process of nixtamalization. Bioavailability of calcium is, however, compromised by phytate, which is present in large quantities in maize kernels and is only modestly reduced by nixtamalization.

Objective: The objective was to compare the absorption of calcium from tortilla meals prepared from low-phytate maize with that from meals prepared from maize with typical phytate content.

Design: At 1-mo intervals, 5 healthy adult women were fed 2 test meals of \( \approx 140 \) g tortillas in lieu of breakfast. On one occasion, the tortillas were prepared from maize with \( \approx 60\% \) phytate reduction, and, on the other occasion, they were prepared from the matching isohybrid wild-type maize. Beginning midway through the test meal, \( ^{44}\text{Ca} \) (0.3 mg/kg body wt) was administered in water as an extrinsic label; \( ^{42}\text{Ca} \) (0.06 mg/kg body wt) was administered intravenously immediately after the test meal. Isotope ratios of \( ^{42}\text{Ca} \) to \( ^{43}\text{Ca} \) and of \( ^{44}\text{Ca} \) to \( ^{43}\text{Ca} \) were measured by inductively coupled plasma mass spectrometry in urine collected as an 8-h pool from the period 16–24 h after intravenous tracer administration and prepared by the oxalate precipitation method. Fractional absorption of calcium was determined by using a dual-isotope ratio technique.

Results: Mean fractional absorption of calcium from tortillas prepared from the low-phytate maize (0.50 ± 0.03) was significantly (\( P = 0.003 \)) greater than that from tortillas prepared from the control maize (0.35 ± 0.07).

Conclusion: The increase in the quantity of calcium absorbed could be of practical importance for calcium nutriture when the intake of dairy products is limited. Am J Clin Nutr 2005;82:84–7.

KEY WORDS Maize, phytate, low-phytate maize alleles, tortillas, calcium absorption

INTRODUCTION

Cereal grains contain only negligible quantities of calcium. However, substantial quantities of this mineral are added to maize during the process of nixtamalization, or liming, which is the traditional method of processing maize in the preparation of tortillas in Central America (1). From the perspective of calcium nutriture, nixtamalization can be regarded as an early example of mineral fortification of a major food staple in Central America. Depending on the extent to which this added calcium is bioavailable, maize tortillas may provide a major source of calcium for persons whose intake of dairy products is limited.

Whereas the absorption of endogenous iron from a low-phytate acid maize was modestly higher than that from control maize (2), substitution of a low-phytate-acid maize has not been associated with any increase in the absorption of iron that was added to maize flour as a fortificant (3). These observations have given additional impetus to determining whether the substitution of low-phytate-acid maize affects the bioavailability of calcium in maize tortillas that is derived almost entirely from the process of nixtamalization.

The hypothesis tested in this study was that fractional calcium absorption (FCA) from tortilla meals prepared from maize with \( \approx 60\% \) phytate reduction is significantly greater than from tortilla meals prepared from wild-type control maize.

SUBJECTS AND METHODS

Study design

Fractional absorption of an extrinsic calcium stable isotope label by healthy adults was measured when administered with a test meal of tortillas prepared from low-phytate maize. In a crossover design with a washout period of \( \geq 4 \) wk, fractional calcium absorption from a test meal of tortillas prepared from a low-phytate maize was compared with that from a tortilla test meal prepared from the isohybrid wild-type control maize.

Calcium absorption was measured using an extrinsic calcium stable isotope label by a dual-isotope tracer ratio technique based...
on measurements in the urine of the dose-adjusted ratio of enrichment with the oral extrinsic label to that for a second calcium stable isotope tracer administered intravenously (4).

Subjects

The subjects were 5 healthy free-living volunteer adult women aged 22–29 y who were recruited from the University of Colorado Health Sciences Center community by word of mouth. We included women of childbearing age who had normal BMI and an omnivore diet and who were willing to eat maize-only breakfasts on 2 consecutive days. Exclusion criteria included pregnancy, lactation, use of a multivitamin or mineral supplement, regular consumption of fortified breakfast cereals or energy bars, regular use of medicines that affect absorption, and the presence of any chronic or acute illness.

Written informed consent was provided by all subjects. The protocol was approved by the Colorado Multiple Institutional Review Board of the University of Colorado Health Sciences Center.

Source of maize and preparation and administration of test meals

Test meals were prepared from low-phytate-acid maize (lpa1-1) that has ≈60% phytate reduction. This maize was provided by Pioneer Hi-Bred Inc (Dupont, Johnston, IA), which cultivated it under a cooperative research and development agreement with the US Department of Agriculture. The isohybrid wild-type maize with normal phytate content was also provided by Pioneer Hi-Bred Inc and was grown in the same location.

For the preparation of tortillas, 10 L water was added to 450 g maize kernels, and the mixture was brought to the boil. Powdered limestone (5 g, or 1 tsp) was added, and the mixture was stirred. The maize mixture was left to simmer for 4 h, after which it was drained and spread out on a towel to dry for 3 h. The nixtamalized maize was then ground in a food processor, rolled into ≈4-cm diameter balls, dipped in corn oil, and flattened. The tortillas were cooked on a greased skillet for 1 min. Each meal consisted of 5 of these tortillas, which weighed ≈35 g.

After an overnight fast, test meals were administered at ≈0800 in the presence of one of the investigators. Three subjects received tortillas prepared from the lpa1-1, and the other 2 received tortillas prepared from the isohybrid wild-type maize. After a washout period of 4 wk, subjects consumed the alternative test meal.

Isotope preparation

Enriched 42Ca and 44Ca stable isotopes were obtained from Trace Science International Inc (Richmond Hill, Canada) as carbonate. Enriched 44Ca was used as the orally administered tracer, and 42Ca was used as the intravenously administered tracer. Calcium carbonate was dissolved by adding drops of concentrated hydrochloric acid. The oral solution was prepared at a calcium concentration of 0.063 mol/L by dilution with Milli-Q water (Millipore Systems, Bedford, MA), and the intravenous solution was prepared at a calcium concentration of 0.01 mol/L. 0.45% sterile sodium chloride. We adjusted the oral solution to pH 5.0 and the intravenous solution to pH 6.0 with sodium hydroxide. The solutions were filtered through a 0.2-μm filter to remove pyrogens. Sterile techniques were used to prepare doses for intravenous administration. Calcium concentrations were determined by atomic absorption spectrophotometry with application of correction factors for atomic weight. The oral solution was stored in plastic tubes, and the intravenous solution was stored in sealed sterile vials. The intravenous dose was tested for pyrogens immediately before use.

Isotope administration

An accurately weighed quantity of 44Ca (≈0.3 mg Ca/kg body wt) was administered orally in water starting approximately halfway through the test meal. We have used this method extensively to administer stable isotope tracers of zinc (5). An accurately weighed quantity of 42Ca (≈0.06 mg Cu/kg body wt) was administered intravenously over a 10-min interval immediately after the test meal. Administration was performed over a 5-min interval with the use of a 10-mL syringe, a 3-way stopcock, and a scalp vein needle placed in a superficial forearm vein. The syringe was flushed twice with sterile normal saline.

Sample collection, preparation, and analysis

Participants were instructed to completely empty the bladder immediately before administration of the intravenous isotope tracer. All urine was collected for an 8-h period beginning 16 h after the administration of the intravenous tracer. Urine was collected directly into an acid-washed plastic bottle. Volumes were measured, and a 50-mL aliquot was then stored at −20 °C.

For analysis, 5 mL urine from the period 16—24 h after isotope administration was purified by the oxalate precipitation method (6). Urine was first centrifuged to remove particulates, saturated ammonium oxalate was adjusted to pH 8.0 with NH4OH, and 1.2 mL saturated ammonium oxalate was added to the urine. After thorough mixing, the sample was left at room temperature overnight. It was then centrifuged at 1700 × g for 15 min at room temperature, and the supernatant fluid was decanted. The precipitate was washed twice with Milli-Q water and dissolved in 4 mL of 2% HNO3. We prepared 8 mL of 1 ppm calcium solution in 2% HNO3 from each sample. Isotope ratios of 42Ca to 43Ca and 44Ca to 43Ca were measured with the use of an inductively coupled plasma mass spectrometer (ICP-MS) (Plasma Quad 3; VG Elemental, Winsford, United Kingdom). Each sample was introduced into the ICP-MS by using an autosampler (ASX-500 Model 510; CETAC, Omaha, NE) and peristaltic pump (Perimax 12; CPETEC, Erding, Germany). To minimize argon-derived isobaric and polyatomic interference, the instrument was operated in the cool plasma mode. Instrumental settings for cool-plasma-mode operation are given in Table 1.

Two-percent HNO3 and a 1 ppm natural abundance calcium standard were used to optimize ICP-MS tuning to attain the lowest ion count for the 2% HNO3 blank and the highest count rate for the natural abundance calcium standard. With a 44Ca count rate of 300 000—400 000 per second from the 1 ppm natural abundance calcium standard, the ICP-MS can be tuned to produce a 2% HNO3 signal of <0.4% of the 1 ppm calcium signal for isotopes 42Ca, 43Ca, and 44Ca. A natural abundance calcium standard was analyzed after every 6 urine samples, and 2% HNO3 was analyzed after every 12 samples. The results were used to reduce any effect of instrumental drift on measured ratios. The 2% HNO3 count rate was subtracted from urine sample count rates. The relative SD for the analysis of 10 replicates was <0.3% for both 42Ca:43Ca and 44Ca:43Ca. The calcium tracer enrichments were calculated from the measured isotope ratios by using
...an algorithm that takes into account the contribution of each isotope signal of calcium from both isotopically enriched tracers and the naturally occurring calcium in the sample. For each isotope tracer used, enrichment is defined as all the calcium in the sample from that particular tracer divided by the total amount of calcium in the sample.

The weights of the test meals consumed were recorded. Tortillas from each test meal were collected and homogenized. Weighed aliquots were digested by heating samples to 450 °C for 24 h, wet ashing on a hot plate with concentrated HNO₃, and then ashing at 450 °C for an additional 24 h, and the calcium content was determined by atomic absorption spectrophotometry (7). HPLC was used for direct measurement of phytate (8).

**Data processing and statistical analysis**

Data were analyzed by using GRAPHPAD PRISM for WINDOWS software (version 4.00; GraphPad Software, San Diego, CA; www.graphpad.com). Mean (±SD) calcium intake was calculated per gram of tortilla and per test meal. The molar calcium:phytate of the test and control meals was calculated.

Mean FCA was determined for the low-phytate and the wild-type maize tortilla meals. Calcium absorption from the low-phytate maize tortilla meals was compared with that from the wild-type control maize tortilla meals by using a two-tailed, paired comparison t-test.

**RESULTS**

Results are presented as means ± SDs. The calcium contents of the low-phytate and control tortillas did not differ significantly (P = 0.90), and the results were combined to give a mean calcium concentration of 1.00 ± 0.39 mg/g wet weight tortilla. The quantity of tortilla consumed averaged 140 g/test meal, and the mean calcium intake was 140 mg. Phytate concentrations in the low-phytate and control tortillas were 1.56 and 3.0 mg/g, respectively, which gave corresponding molar calcium:phytate of 10.9 and 4.9. All individual subjects had significantly higher FCA from the lpa1-1 maize tortilla meals than from the control maize tortilla meals (Figure 1). Mean FCA from the low-phytate maize tortilla meals was 0.50 ± 0.03 compared with a mean of 0.35 ± 0.07 from the tortilla meals prepared from the isohybrid wild-type control maize (P = 0.003).

**DISCUSSION**

Phytate strongly chelates calcium. Calcium-phytate complexes are quite soluble at an acidic pH but have only very limited solubility at the neutral or alkaline pH of the small intestine (9). Phytate was reported as early as 1934 to have an inhibitory effect on calcium absorption in rats (10). Subsequent reports of the effects of phytate on calcium absorption in rodents were conflicting (9–17), which, it was suggested, may be due to the presence of intestinal phytases in the rodent (18). The substitution of low-phytate cereal grains for grains with a typical phytate content in feed for chicks, pigs, and fish has been associated with improved calcium nutrition in each species (19–21). The destruction of phytate in wheat flour was shown in 1942 to improve calcium retention in humans (22). The inhibitory effect of phytate on calcium absorption in humans has subsequently been confirmed (23, 24), and calcium absorption from low-phytate soybeans was reported to be significantly higher than that from high-phytate soybeans (18). Even in humans, however, the adverse effect of phytate on calcium absorption has excited no (25) or relatively little (26) attention in reviews.

In the current study, the difference between the absorption of calcium from tortilla meals prepared with the low-phytate maize and that from meals prepared with maize with a typical phytate content was similar but of greater magnitude than the difference between the absorption of calcium from low-phytate and high-phytate whole cooked soybeans (18). The calcium content of the tortillas prepared for this study was comparable to that found by other investigators (1). With a typical tortilla wet weight of ≈40 g, the quantity of calcium ingested with each tortilla was ≈40 mg. In our own experience in the western highlands of Guatemala, adult women typically consume ≥15–20 tortillas/d (weight: ≈40 g each), which provide ≈500 mg calcium/d. The increased absorption found in the low-phytate maize tortillas would contribute an additional 6 mg Ca absorbed per tortilla or 90–120 mg Ca/d for typical maize intakes. Thus, when there is a limited intake of dairy products, tortillas provide the major source of dietary calcium. This calcium alone, however, is not typically sufficient to match the adequate intake recommended by the Institute of Medicine’s Food and Nutrition Board (27), which states that any strategy that increases the bioavailability of this
calcium will make a useful contribution to calcium status. Even in North America, tortillas prepared from nixtamalized maize can provide a useful alternative source of calcium for subjects who do not consume dairy products, and the superior absorption of calcium from a low-phytate maize could be advantageous.

We showed previously that fractional absorption of zinc from tortillas prepared from maize with 2 different amounts of phytate reduction (but probably a mutation of the same allele) is higher than that from tortillas prepared from the corresponding wild-type maize with typical phytate content (28, 29). Others have found a modest increase in iron absorption (2). Especially when taken together, these data encourage the evaluation of the efficacy and effectiveness of a change in agricultural practice—ie, the use of low-phytate maize—to improve mineral nutriture in low-income populations that depend on maize as their principal food staple.

KMH, NFK, JLW, SL, LVM, and VR participated in study design and data interpretation. SL and KLP were responsible for laboratory analyses. Data were analyzed by JLW. The manuscript was drafted by KMH. None of the authors had any personal or financial conflicts of interest.

REFERENCES

Plasma kinetics of lutein, zeaxanthin, and 3'-dehydro-lutein after multiple oral doses of a lutein supplement1–3

Petra A Thürmann, Wolfgang Schalch, Jean-Claude Aebischer, Ute Tenter, and William Cohn

ABSTRACT

Background: Adequate intake of lutein is postulated to reduce the risk of age-related macular degeneration, but kinetic information for developing a dosing regimen is sparse.

Objective: The objective was to characterize lutein plasma kinetics in a multiple dosing design and to assess the effects of lutein intake on concentrations of other plasma carotenoids.

Design: After a run-in period of 7 d, 19 healthy volunteers were assigned to receive daily oral doses of 4.1 mg lutein (n = 8; group 1) or 20.5 mg lutein (n = 8; group 2) for 42 d or no lutein (n = 3; control group). The supplement contained 8.3% zeaxanthin relative to lutein (100%). The time profiles of plasma xanthophyll concentrations were monitored over the dosing phase, and samples were collected frequently on day 42 and for 24 d after dosing.

Results: Average plasma all-E-lutein concentrations increased from 0.14 to 0.52 ± 0.13 and 1.45 ± 0.69 μmol/L in groups 1 and 2, respectively. Dose-normalized lutein bioavailability in group 2 was ≈60% of that in group 1. Kinetic disposition half-life did not differ significantly between groups. On average, dosing for 18 d was required to reach a >90% fraction of the steady state concentration, which is consistent with an effective half-life for accumulation of ≈5.6 d. Plasma kinetics of all-E-lutein were paralleled by those of all-E-3'-dehydro-lutein. Kinetic analysis indicated formation of all-E-3'-dehydro-lutein from lutein. Lutein was well tolerated and did not affect the concentrations of other carotenoids.

Conclusion: Long-term supplementation with 4.1 and 20.5 mg lutein as beadlets increased plasma lutein concentrations 3.5- and 10-fold, respectively. Am J Clin Nutr 2005;82:88–97.

KEY WORDS Xanthophylls, carotenoids, lutein, zeaxanthin, all-E-3'-dehydro-lutein, multiple oral dose kinetics, macular pigment, age-related macular degeneration

INTRODUCTION

An adequate supply of the dietary carotenoids lutein and zeaxanthin depends on regular intakes of fruit and green and yellow vegetables, which represent major natural sources of xanthophylls (1). The combined daily intake of lutein plus zeaxanthin ranges, on average, between 2 and 26 mg, for which a lutein-to-zeaxanthin ratio of ≈5:1 is generally assumed (2–4). In the human eye, lutein and zeaxanthin are specifically located in the center of the retina, where they form the “yellow spot,” or macula lutea (5). Because of this specific location and their physicochemical properties, such as the absorption of high-energy blue light and their capability to quench reactive oxygen species, a protective action of the xanthophylls in the retina has been postulated (6). Evidence indicates that a low intake of lutein and zeaxanthin is related to an increased risk of age-related macular degeneration (7, 8). Moreover, an increased intake of lutein and zeaxanthin appears to be associated with a lower risk of cataract (9, 10). Although the supplemental intake of lutein, zeaxanthin, or both has been suggested to be useful in persons at high risk of macular degeneration (11–13), critical comment asks for additional prospective trials before general recommendations should be made (14).

The development of appropriate dosing regimens depends on adequate information on the pharmacokinetic properties of a compound. However, little is known about the kinetics of lutein. Several studies have dealt with the comparative availability of lutein in plasma, providing xanthophyll as lutein or as lutein ester in various foodstuffs or in formulated supplements (15–18). Landrum et al (19) treated 2 volunteers with a 30-mg daily dose for 140 d. Plasma concentrations plateaued after 20–40 d, with a 10-fold increase from baseline, and returned to baseline concentrations 40–50 d after supplementation was discontinued. Depletion studies estimate the terminal half-life of lutein to be ≈15 d in patients with type 1 diabetes (20) and to be ≈76 d in healthy subjects (21).

We recently reported on the pharmacokinetics of zeaxanthin after multiple dosing in healthy subjects and showed that all-E-3'-dehydro-lutein is formed from zeaxanthin (22). The present study aimed to acquire additional kinetic data on lutein in a multiple-dose design study in healthy subjects. The dose was chosen to provide an amount in the range of ≈2- to 4-fold the average daily intake (4.1 mg/d; low dose) and to facilitate the assessment of pharmacokinetic parameters (20.5 mg/d; high dose). The kinetic data to be generated were plasma concentration-dose response at steady state, time to attain steady state, index of accumulation, effective half-life for accumulation, and dose proportionality. Moreover, the plasma kinetics of all-E-3'-dehydro-lutein (3R,6R-3-hydroxy-β-carotene-3'-one) and its

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Subjects and Methods

Subjects

Nineteen healthy men and women gave their written informed consent to participate in this monocentric, randomized, open-label, parallel-group study. All subjects were healthy according to the results of a physical examination that included blood pressure and heart rate measurements, an electrocardiogram, and clinical chemistry, hematology, and serology (hepatitis B and C, HIV-1, and HIV-2) tests. Written informed consent was obtained from each subject after they received an adequate explanation of the aims, methods, objectives, and potential hazards of the study. The study protocol was in accordance with the German drug law and the Declaration of Helsinki and was approved by the local ethics committee of the University of Witten/Herdecke, Germany. Demographic data and information on nutritional habits are provided in Table 1.

Study design

Lutein, extracted from marigold, was formulated in beadlets (5% Lutein, tablet grade; DSM Nutritional Products, Ltd), which were incorporated in hard gelatin capsules containing 4.1 mg lutein (of which 93% was all-E-lutein) and 0.34 mg was zeaxanthin (of which 97% was all-E-zeaxanthin).

Eight subjects (4 women, 4 men) were randomly assigned to receive 4.1 mg lutein/d (one capsule; group 1), 8 (4 women, 4 men) were randomly assigned to receive 20.5 mg lutein/d (5 capsules; group 2), and 3 (2 women, 1 man) were randomly assigned to served as control subjects to estimate baseline fluctuations of plasma lutein and zeaxanthin concentrations. For the entire study, subjects were asked to avoid lutein- and zeaxanthin-rich vegetables and fruit such as kale, Brassica oleracea, spinach, carrots, corn, tomatoes, nectarines, and peaches. Daily fat intake was restricted to 100 g. The subjects received nutritional diaries and were required to complete a 1-d dietary record thrice weekly. The capsules were ingested with 150 mL water and a light breakfast. Compliance was controlled by counting the remaining capsules at each visit.

On day 67 a final physical examination and laboratory tests were performed following the same protocol used at the inclusion visit. After a 1-wk run-in period, blood samples for the measurement of baseline plasma lutein concentrations were drawn on 3 consecutive d and thereafter at weekly intervals until week 5 (day 35). Additional blood samples were taken on days 38, 39, 40, and 41. Except for the blood specimens collected on day 42, all blood specimens were drawn in the morning, before dosing, after the subjects had fasted overnight. On day 42 blood samples were obtained before dosing and 2, 4, 6, 8, 12, and 24 h after dosing (24-h kinetic profile). On this “pharmacokinetic study day,” the capsules were taken with a standardized breakfast consisting of

### Table 1

Demographic data and nutritional habits of the subjects

<table>
<thead>
<tr>
<th></th>
<th>Group 1†</th>
<th>Group 2‡</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 8)</td>
<td>(n = 8)</td>
<td>(n = 3)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>28.6 ± 7.9 (26.5)</td>
<td>28.6 ± 4.8 (28.0)</td>
<td>38.7 ± 6.7 (37.0)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>76.9 ± 14.3 (78.8)</td>
<td>72.3 ± 7.4 (74.5)</td>
<td>61.0 ± 5.5 (60.0)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>172.5 ± 6.9 (173.5)</td>
<td>175.8 ± 5.5 (176.0)</td>
<td>168.3 ± 6.7 (170.0)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.7 ± 3.5 (25.5)</td>
<td>23.4 ± 2.3 (23.2)</td>
<td>21.5 ± 0.7 (21.6)</td>
</tr>
<tr>
<td>Energy intake (kcal/d)</td>
<td>1862 ± 616 (1835)</td>
<td>2051 ± 851 (1894)</td>
<td>1716 ± 567 (2040)</td>
</tr>
<tr>
<td>Fat intake (g/d)</td>
<td>77 ± 33 (71)</td>
<td>79 ± 39 (75)</td>
<td>74 ± 25 (71)</td>
</tr>
<tr>
<td>Dietary lutein intake (mg/d)</td>
<td>0.552 ± 0.726 (0.300)</td>
<td>0.753 ± 0.205 (0.240)</td>
<td>1.295 ± 2.208 (0.487)</td>
</tr>
</tbody>
</table>

† All values are x ± SD; medians in parentheses. There were no significant differences between groups, P > 0.05.

‡ Low-dose group: 4.1 mg lutein/d.

§ High-dose group: 20.5 mg lutein/d.
one roll of bread with cheese and coffee or tea. In the postdosing period, additional blood samples were drawn in the morning on days 43, 44, 48, 53, 58, 62, and 67. For the control subjects, blood sampling was performed only at the end of the run-in period (3 samples) and on days 28, 48, and 67.

Blood samples of 7.5 mL each were drawn into precooled monovettes (Sarstedt, Nuembrecht, Germany) containing EDTA and immediately centrifuged for 10 min at 4 °C and 2500 × g. Plasma was separated under light protection, immediately transferred into polypropylene tubes, and stored at −35 °C.

Analytic methods

Plasma samples were analyzed for the E and Z isomers of lutein, all-E-3'-dehydro-lutein, and all-E-zeaxanthin and for the sums of Z-zeaxanthin isomers (typically: 85% 13-Z-zeaxanthin, 9-Z-zeaxanthin, and 15-Z-zeaxanthin) and Z-lutein isomers (9-Z-lutein, 9'-Z-lutein, 13-Z-lutein, 13'-Z-lutein, and 15-Z-lutein isomers) as previously described (22). Recovery rates of lutein and zeaxanthin were 100% and 99%, respectively. Plasma concentrations in the range 0.005–2.8 μmol/L could be measured with intraday CVs of 4.8% (lutein) and 5.6% (zeaxanthin) and interday CVs of 2.6% (lutein) and 6.8% (zeaxanthin), respectively. The lower limit of detection was 0.002 μmol/L; the lower limit of quantification was 0.007 ± 0.002 μmol/L.

β-Carotene, lycopene, β-cryptoxanthin, α-carotene, and retinol concentrations were measured according to Aebischer et al (25). Cholesterol and triacylglycerol concentrations in plasma were measured according to Richmond (26), Fossati and Prencipe (27), and McGowan et al (28) by using the CHOD-PAP method (Merck AG, Dietikon, Switzerland), adapted to a centrifugal analyzer (Cobas-Bio, Roche Diagnostics, Basel, Switzerland).

Pharmacokinetic analyses

Lutein and zeaxanthin

Baseline plasma xanthophyll concentrations (C_0) were calculated individually as the mean value of the 3 predosing samples on days −3 to −1. The approach to plateau was monitored by recording lutein or zeaxanthin trough concentrations over the dosing period, and the corresponding plasma concentration-time profiles were modeled according to an equation of the same form as that describing the kinetics following a constant-rate intravenous infusion (29, 30). The data were fitted according to the following equation:

\[ C(t) = H \times (1 - e^{-k_{eff} \times t}) + C_B \]  

where \( C(t) \) is the predicted xanthophyll concentration at time \( t \), \( H \) is the concentration increase to steady state, and \( k_{eff} \) is the rate constant; the latter 2 variables were estimated by fitting as described below. The half-life associated with \( k_{eff} \) was calculated as \( t_{1/2} = \ln(2)/k_{eff} \).

Steady state parameters were derived from the time concentration profiles over a dosing interval \( \tau \) (24 h) recorded at day 42. The primary parameters included the area under the plasma concentration-time curve (AUC) and the corresponding baseline-corrected \( \Delta \)AUC, both calculated by the linear trapezoidal rule (29, 30). The steady state concentration \( (C_{ss}) \) is defined as the time-averaged concentration over the dosing interval and was calculated as \( \text{AUC}/\tau \). The maximum baseline-corrected plasma concentrations after a dose \( (C_{max}) \), the time to reach maximum concentrations \( (t_{max}) \), and the predose baseline-corrected concentrations \( (C_{min}) \) were derived directly from the observed data. Baseline-corrected steady state concentration was defined as the average concentration over the dosing interval \( \tau \) and calculated as \( \Delta C_{ss} = \Delta \text{AUC}/\tau \). An additional secondary parameter was the peak-trough fluctuation [PTF \( = (\Delta C_{max} - \Delta C_{min})/\Delta C_{ss} \) (30). For the comparison of the 2 dosing groups, \( \Delta C_{ss}, \Delta \text{AUC}, \Delta C_{max}, \text{and} \Delta C_{min} \) were dose-normalized by dividing these parameters by the corresponding daily doses (in μmol).

Postdosing concentration-time profiles were best fitted according to the monoexponential plus constant model represented by the following equation:

\[ C(t) = A \times e^{-k_d \times t} + B \]  

where \( A \) is the preexponential coefficient, \( k_d \) is the apparent disposition rate constant, and \( B \) is a constant. Competing models were the monoexponential and the biexponential model in addition to elimination according to a Michaelis-Menten mechanism. Model selection was based on parameter precision, inspection of weighted residuals, and parsimony criteria by formally testing for nonrandomness of errors as judged by run tests, the \( F \) ratio test (for nested models), the Akaike information criterion, and the Schwarz criterion (31, 32).

For the decrease in plasma concentrations of all-E-lutein and -zeaxanthin after day 42, the best fit could be achieved by the monoexponential plus constant model for all subjects in group 2. Because the concentration dose response was much lower for group 1, which affected the sensitivity for model discrimination, kinetic profiles of competing models were not tested for goodness of fit and model order. The half-life associated with \( k_d \) was calculated as \( t_{1/2,eff} = \ln(2)/k_{eff} \).

The accumulation index \( R \) was calculated as follows:

\[ R = 1/(1 - e^{-k_d \times \tau}) \]  

For the kinetic analysis, plasma xanthophyll concentrations were fitted to models by using nonlinear least-squares regression analysis (WINSAAM, version 3.03; NIH, Bethesda, MD) (33). Measurement errors were assumed to be independent and normally distributed with a mean of 0 and a fractional SD of 0.05. Weights were chosen equal to the inverse of the variance of the measurement error. The SE of the parameter estimates was determined from the covariance matrix of the least-squares fit and was expressed as the CV.

Attainment of steady state concentrations was detected by monitoring the predose concentration-time data for each subject by linear regression for the interval from day 38 to day 43. Steady state was assumed to be reached, provided that the slopes of individual regression lines were statistically not distinct from 0.

3'-Dehydro-lutein

Baseline concentrations of 3'-dehydro-lutein and the baseline-corrected concentrations \( \Delta C_{max}, \Delta \text{AUC}, \text{and} \Delta C_{ss} \) were evaluated as described for lutein and zeaxanthin. A model for the formation and elimination of 3'-dehydro-lutein was postulated, assuming that the plasma concentration of this carotenoid was metabolically derived from plasma lutein. To probe for such a precursor-product interrelation, a precursor time course was generated in WINSAAM by using a forcing function for lutein. This functional description of the plasma lutein data was provided by
linear interpolation between sequential pairs of data, recreating the shape of the input system and driving the formation of 3'-dehydro-lutein (22).

The formation and elimination of 3'-dehydro-lutein was modeled by the following differential equation:

$$\frac{dC_{DHL}}{dt} = k_f \times \Delta C_{lutein} - k_e \times (C_{DHL} - B_{DHL}) \quad (4)$$

where $d$ is a derivative, $\Delta C_{lutein}$ is the baseline-corrected concentrations of lutein, $C_{DHL}$ is the plasma concentration of 3'-dehydro-lutein, and $B_{DHL}$ is the 3'-dehydro-lutein baseline concentration. The rate constants for 3'-dehydro-lutein formation and elimination are designated as $k_f$ and $k_e$, respectively. Data fitting according to this precursor-product model was carried out for the pair all-E-lutein and all-E-3'-dehydro-lutein; $k_f$, $k_e$, and $B_{DHL}$ were estimated by nonlinear least-squares regression analysis (WINSAAM, version 3.03) as outlined above.

Statistical analysis

Pharmacokinetic parameters of total lutein, all-E-lutein, zeaxanthin, and 3'-dehydro-lutein are presented as means ± 1 SDs. For $t_{\text{max}}$ and the various half-lives, arithmetic means are given. Geometric means and corresponding SDs were calculated for $\Delta C_{\text{ss}}$, $\Delta C_{\text{max}}$, $C_{\text{min}}$, AU, and PTF, assuming a logarithmic normal distribution (as justified in reference 34).

Effects caused by interactions between sex and treatment were tested by ANOVA. In the absence of any significant sex effects, parameters were compared among treatment groups by one-way ANOVA (procedure aov, S-PLUS, version 6; Insightful Corporation, Seattle, WA). Effects were evaluated as differences in arithmetic means (group 1 – group 2; including baseline concentrations and half-life) or as the ratio of means (group 1/group 2) for natural log-transformed parameters (including dose-normalized $\Delta C_{\text{ss}}$ and $\Delta C_{\text{max}}$, $R$, and PTF). Two-sided 95% CIs were constructed with the $t$ distribution by using the square root of the residual variance in the analysis of variance table; $t_{\text{max}}$ was analyzed by applying the Mann-Whitney $U$ test. To compare effects within the same subject, further differences were evaluated by paired $t$ tests. Attainment of steady state was tested by examining whether the zero value was contained in the 95% CI of the regression slopes. The level of statistical significance was set to $P < 0.05$ for all tests. All additional calculations were computed by using MATHCAD 2000 (MathSoft, Cambridge, MA).

RESULTS

All subjects completed the study according to the trial protocol, and no adverse events occurred that were related to lutein supplementation. Compliance was excellent (97–100%). Although the subjects in this study were required to avoid the intake of lutein-rich vegetables and fruit, the residual lutein intake was determined by differing eating habits. Thus, average dietary lutein consumption was highly variable, but median values were lower in all groups (Table 1) compared with data from nutritional surveys that showed a population mean value of 1–2 mg lutein/d. This finding indicates that the subjects complied with the dietary restrictions of the present study.

Plasma kinetics of all-E-lutein and all-E-zeaxanthin

Mean concentrations of all-E-lutein increased to plateau concentrations in response to administration of multiple oral doses of 4.1 mg lutein (group 1) or 20.5 mg lutein (group 2) and subsequently decreased after the cessation of dosing on day 42 (Figure 2A). all-E-Lutein represented the major fraction of lutein throughout the study, contributing to ~85% at baseline and during the postdosing period and to ~90% during dosing (data not shown). The remaining lutein contributions were determined as the sum of the 9-Z, 9'-Z, 13-Z, 13'-Z, and 15-Z isomers. Concentration-time profiles for all-E-lutein on day 42 are presented in Figure 2B. Control subjects did not ingest lutein supplements; therefore, baseline plasma all-E-lutein concentrations were maintained (Figure 2A).

Attainment of lutein steady state was tested by evaluating the plasma concentration profile of predose samples (ie, blood specimens collected at the end of a dosing interval and just before ingestion of the next dose) over days 38–43 (Figure 3) by linear regression. The resulting mean slopes were 0.010 ± 0.031 and 0.003 ± 0.027 for groups 1 and 2, respectively, ie, statistically not distinct from 0. This finding indicated that steady state had been reached at day 38 or before.

In addition to lutein, the dosage form contained a small amount of zeaxanthin (8.3% with respect to lutein). Although the kinetic profiles for all-E-zeaxanthin are not presented, the kinetic parameters for both all-E-xanthophylls are summarized in Table 2. Baseline plasma all-E-lutein and all-E-zeaxanthin concentrations were not significantly different between dosing groups. On day 42 peak xanthophyll plasma concentrations were reached ~10–11 h after dosing. There was little fluctuation in lutein and zeaxanthin concentration on day 42 (Figure 2B), as evident from
PTF values (Table 2). Steady state all-E-lutein concentrations exceeded baseline concentrations by ≈3.5- and 10-fold for groups 1 and 2, respectively. The increases from baseline to steady state concentrations and the corresponding ΔC\textsubscript{max} were consistently greater in group 2 than in group 1 for both xanthophylls. The two-sided 95% CIs for the ratio of the geometric means (group 1/group 2) for all-E-lutein ranged from 0.19 to 0.45 and from 0.21 to 0.50 for ΔC\textsubscript{ss} and ΔC\textsubscript{max}, respectively. The corresponding 95% CIs for all-E-zeaxanthin were 0.17–0.42 and 0.30–0.46, respectively. The steady state concentrations of the sums the E- and Z-lutein isomers were 0.59 ± 0.14 and 1.64 ± 0.77 μmol/L for groups 1 and 2, respectively.

Dose proportionality was examined by comparing dose-normalized ΔC\textsubscript{max} and ΔC\textsubscript{ss} values between group 1 and group 2. Dose-normalized ΔC\textsubscript{ss} and ΔC\textsubscript{max} values for all-E-lutein were significantly higher in the low-dose group (0.054 ± 0.017 and 0.063 ± 0.027 L\textsuperscript{−1}, respectively) than in the high-dose group (0.036 ± 0.016 and 0.039 ± 0.018 L\textsuperscript{−1}, respectively). The pertaining two-sided 95% CI for the ratio of geometric means (group 1/group 2) ranged from 1.01 to 2.25 and from 1.02 to 2.52 for ΔC\textsubscript{ss} and ΔC\textsubscript{max}, respectively. For all-E-zeaxanthin, dose-normalized incremental, steady state concentrations did not differ significantly between groups 1 and 2 (ΔC\textsubscript{ss}: 0.045 ± 0.020 and 0.034 ± 0.016 L\textsuperscript{−1}, respectively); the 95% CI for the ratio of geometric means (group 1/group 2) ranged from 0.83 to 2.10. Similarly, dose-normalized ΔC\textsubscript{max} values for all-E-zeaxanthin concentrations did not differ significantly between groups 1 and 2 (0.058 ± 0.022 and 0.039 ± 0.018 L\textsuperscript{−1}, respectively). The corresponding 95% CI for the ratio of geometric means (group1/group 2) ranged from 0.96 to 2.29.

The cholesterol-normalized concentration-time profiles of lutein resembled those shown in Figure 2A and had similar intersubject variations (data not shown). As described in Subjects and Methods, empirical modeling was used to fit both the time courses of the approach to plateau in response to carotenoid dosing and the decay of plasma concentrations during the post-dosing phase (Figure 4). The monoexponential plus constant model was applied to fit the decay curves of all-E-lutein in both groups and for all-E-zeaxanthin in the high-dose group. Model parameters for all-E-zeaxanthin in the low-dose group could not be accurately estimated because the increase in plasma concentrations above baseline was too small. The precision of all-E-lutein disposition half-life estimates for each subject was acceptable, because the CVs were <20% and 12% for groups 1 and 2, respectively. The half-life of apparent lutein disposition (t\textsubscript{d}) for all-E-lutein and all-E-zeaxanthin ranged between 5 and 7 d (Table 2), and the half-lives for all-E lutein were not significantly different between groups. Again, the precision of the parameters characterizing all-E-lutein plasma accumulation for each subject was acceptable, because the CVs were <16% and 10% for groups 1 and 2, respectively. However, for 2 subjects in group 1 and for 1 subject in Group 2, at least one kinetic parameter resulted in 95% CIs, which included 0, and thus by inference, the

**TABLE 2**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (μmol/d)</td>
<td>6.703</td>
<td>33.513</td>
</tr>
<tr>
<td>C\textsubscript{ss} (μmol/L)</td>
<td>0.140 ± 0.031</td>
<td>1.048 ± 0.120</td>
</tr>
<tr>
<td>ΔC\textsubscript{max} (μmol/L)</td>
<td>0.425 ± 0.179</td>
<td>1.320 ± 0.594⁴⁻⁵</td>
</tr>
<tr>
<td>t\textsubscript{max} (d)</td>
<td>9.8 ± 2.4</td>
<td>11.3 ± 4.6</td>
</tr>
<tr>
<td>ΔAUC (μmol · h/L)</td>
<td>9.105 ± 2.904</td>
<td>30.200 ± 13.392⁴⁻⁵</td>
</tr>
<tr>
<td>ΔC\textsubscript{SS} (μmol/L)</td>
<td>0.362 ± 0.112</td>
<td>1.200 ± 0.551⁴⁻⁵</td>
</tr>
<tr>
<td>PTF</td>
<td>0.267 ± 0.213</td>
<td>0.220 ± 0.108</td>
</tr>
<tr>
<td>C\textsubscript{ss} (μmol/L)</td>
<td>0.517 ± 0.129</td>
<td>1.452 ± 0.689⁴</td>
</tr>
<tr>
<td>t\textsubscript{eff} (d)</td>
<td>4.58 ± 0.38</td>
<td>6.71 ± 1.50⁶</td>
</tr>
<tr>
<td>t\textsubscript{d} (d)</td>
<td>5.49 ± 2.12</td>
<td>6.09 ± 0.98</td>
</tr>
<tr>
<td>R²</td>
<td>7.11 ± 0.55</td>
<td>9.97 ± 2.32⁴</td>
</tr>
</tbody>
</table>

¹ n = 8 per group, except for t\textsubscript{eff} (n = 6 for group 1 and n = 7 for group 2). Data for both sexes were pooled. C\textsubscript{ss}, baseline concentration; ΔC\textsubscript{max}, baseline-corrected maximum concentration on day 42; t\textsubscript{max}, time to reach maximum concentration on day 42; ΔAUC, baseline-corrected area under the concentration-time curve over 24 h on day 42; ΔC\textsubscript{SS}, baseline-corrected steady state concentration on day 42; PTF, peak-trough fluctuation; t\textsubscript{eff}, efficient half-life of accumulation; C\textsubscript{ss}, steady state concentration; t\textsubscript{d}, apparent disposition half-life; R², accumulation index; ND, not determined.

² Arithmetic x ± SD (including data for both sexes).

³ Geometric x ± SD.

⁴ Significantly different from group 1, P < 0.05 (ANOVA).

⁵ Corresponding dose-normalized data differ for the 2 groups, P < 0.05.
parameter was not established. Data for these subjects were not considered for further data compilation in Table 2. The mean half-lives of accumulation ($t_{\text{eff}}$) for plasma all-$E$-lutein were, in a formal statistical sense, different between groups 1 and 2, because the 95% CI for the arithmetic mean differences between groups were $-3.53$ and $-0.73$ d, respectively. This finding indicated that 15 (group 1) or 22 (group 2) d were required to reach 90% of steady state concentrations. Accordingly, the calculated accumulation factors for all-$E$-lutein differed between groups. By contrast, the $t_{\text{d}}$ values for all-$E$-lutein were not significant different between groups (95% CI for corresponding mean differences: $-2.37$, 1.17 d).

As shown by paired $t$ tests, half-lives determined from lutein accumulation ($t_{\text{eff}}$) were consistently not different from those from lutein plasma decay ($t_{\text{d}}$) for group 1 (95% CI: $-0.54$, 0.38 d) and group 2 (95% CI: $-0.57$ to 1.54 d) and for the pooled data from both groups (95% CI: $-0.32$, 0.77 d).

Mean values of the all-$E$-lutein parameter $B$, representing the constant of the kinetic disposition model, were $0.158 \pm 0.062$ and $0.226 \pm 0.223 \mu$mol/L for groups 1 and 2, respectively, and consistently exceeded baseline concentrations (Table 2). However, the difference between constant term $B$ and basal concentrations accounted to $\approx 8 \pm 5\%$ of the incremental dose response for group 2, and these differences were significant (paired $t$ test) on the basis of the corresponding 95% CIs for the geometric mean ratios (baseline concentration/parameter $B$) for group 1 (0.60, 1.24) and group 2 (0.49, 0.70), respectively.

Baseline lutein concentrations are, to some extent, a predictor of the increase in lutein from baseline to steady state plasma concentrations, as shown in Figure 5. To emphasize the sigmoid behavior, data were fitted according to a Hill equation (data for group 2 only). There was a significant positive correlation between the dose-normalized increments in plasma all-$E$-lutein concentrations and the dose-normalized increase in plasma all-$E$-zeaxanthin concentrations ($r^2 = 0.68$, $P < 0.05$; data not shown).

**Plasma kinetics of all-$E$-3'-dehydro-lutein**

After lutein dosing, lutein concentrations of all-$E$-3'-dehydro-lutein increased significantly by factors of 1.7 (group 1) and 4.2 (group 2) over mean baseline concentrations, as shown by paired $t$ tests. (Figure 6A). Kinetic parameters of all-$E$-3'-dehydro-lutein are summarized in Table 3. The plasma concentration time profiles on day 42 were unvarying for both dosing groups (Figure 6B). The dose-normalized $\Delta C_{\text{max}}$ and $\Delta AUC$ were not significantly different between groups 1 and 2.

Because the rise in plasma lutein concentrations was paralleled by an increase in all-$E$-3'-dehydro-lutein concentrations, a
possible parent-compound metabolite relation was investigated, assuming that the rate of 3'-dehydro-lutein formation is proportional to the lutein plasma concentration. This amounted to postulating first-order kinetics (ie, one step or a series of first-order reactions) for the conversion. For 2 subjects (out of 8) in group 1, at least one kinetic parameter resulted in 95% CIs that included 0, and, thus, by inference, the parameter was not established. The parameters for those 2 subjects were not considered for further data compilation in Table 3. The precision of the parameters for individuals in group 2 was adequate; the CV was 7% for parameters $k_t$ and $k_e$. Because the increase in 3'-dehydro-lutein above baseline was not substantial for group 1, a somewhat lower individual precision was found, which was still adequate for $k_e$ (CV: 20%) and $k_t$ (CV: 22%). The kinetic parameters $k_t$ and $k_e$ and the related half-lives did not differ significantly between groups. Further evidence for a joint increase in all-E-lutein and all-E-3'-dehydro-lutein concentrations was established by the highly significant correlation between baseline-corrected steady state plasma concentrations of these compounds on day 42 (Figure 7).

### Plasma concentrations of other carotenoids and retinol

To establish whether or not there was an effect of xanthophyll dosing on concentrations of other carotenoids and retinol, mean baseline concentrations values and time-averaged (over 24 h) $C_{\text{ss}}$ concentrations on day 42 were compared. The sum of the $E$ and $Z$ isomers of lycopene, $\alpha$- and $\beta$-carotene, and $\beta$-cryptoxanthin before dosing and at the end of the dosing phase (day 42) are presented in Table 4. Lutein dosing did not affect concentrations of these compounds because there were no significant differences between baseline and steady state concentrations. The baseline concentration of $\beta$-cryptoxanthin in group 1 exceeded that of group 2 (arithmetic difference: 0.181; 95% CI: 0.027, 0.336). There were no further differences between baseline or $C_{\text{ss}}$ values between groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{ss}}$ (nmol/L)</td>
<td>38.0 ± 9.5</td>
<td>34.3 ± 22.7</td>
</tr>
<tr>
<td>$\Delta C_{\text{max}}$ (nmol/L)</td>
<td>26.1 ± 11.5</td>
<td>110.3 ± 59.8</td>
</tr>
<tr>
<td>$t_{\text{max}}$ (h)</td>
<td>1.3 ± 1.4</td>
<td>3.8 ± 8.9</td>
</tr>
<tr>
<td>$\Delta \text{AUC} (\mu\text{mol} \cdot \text{h} / \text{L})$</td>
<td>0.519 ± 0.283</td>
<td>2.516 ± 1.382</td>
</tr>
<tr>
<td>$C_{\text{ss}}$ (nmol/L)</td>
<td>60.0 ± 12.0</td>
<td>128.7 ± 25.7</td>
</tr>
<tr>
<td>$t_{1/2}$ (d)</td>
<td>3.58 ± 1.85</td>
<td>3.26 ± 0.74</td>
</tr>
<tr>
<td>$k_t$ (d$^{-1}$)</td>
<td>0.018 ± 0.011</td>
<td>0.017 ± 0.004</td>
</tr>
<tr>
<td>$k_e$ (d$^{-1}$)</td>
<td>0.258 ± 0.159</td>
<td>0.222 ± 0.048</td>
</tr>
</tbody>
</table>

$^1$ All values are $\bar{x} \pm \text{SD}; n = 8$ per group, except for $t_{1/2}$, $k_t$, and $k_e$ ($n = 6$ for group 1 and $n = 8$ for group 2). Corresponding data for both sexes were pooled. $t_{1/2}$, terminal elimination half-life; $k_t$, formation rate constant; $k_e$, elimination rate constant; $C_{\text{ss}}$, steady state concentration; $C_{\text{n}}$, baseline concentration; $\Delta C_{\text{max}}$, baseline-corrected maximum concentration on day 42; $t_{\text{max}}$, time to reach maximum concentration on day 42; $\Delta \text{AUC}$, baseline-corrected area under the concentration-time curve over 24 h on day 42.

2 Arithmetic $\bar{x} \pm 1$ SD.

3 Geometric $\bar{x} \pm$ SD.

4 Significantly different from group 1, $P < 0.05$ (ANOVA).

### DISCUSSION

The findings of the present study complement those of a previous trial of the chemically related zeaxanthin (22), ie, the pharmacokinetics of lutein and zeaxanthin showed many similarities. The inclusion of a small fraction of zeaxanthin into the dosing preparation provided an opportunity to study the kinetics of this carotenoid under conditions of lutein excess. In the absence of supplemented lutein, dose-normalized $\Delta C_{\text{ss}}$ values for all-E-zeaxanthin were 0.086 and 0.050 L$^{-1}$ for zeaxanthin doses of 1.76 and 17.6 $\mu$mol/d (22), which exceeded the dose-normalized concentrations of 0.045 and 0.034 L$^{-1}$ observed for group 1 (dose $= 0.58 \mu$mol) and group 2 (dose $= 2.90 \mu$mol) in the present investigation. Therefore, lutein dosing impaired zeaxanthin bioavailability but did not affect plasma concentrations of other carotenoids and retinol (Table 4). Lutein was shown previously to reduce $\beta$-carotene absorption in single-dose studies (35–37), but such an effect was not manifested in the present multiple-dose study.

Evidence for the attainment of apparent steady state was 2-fold: 1) predose samples from days 38 to 43 (Figure 3) indicated that plasma lutein concentrations were maintained, and 2) based on $t_{\text{max}}$ values (Table 2), the calculated fraction of steady state was $>99\%$ for this interval. The time required to attain a $>90\%$ fraction of plasma steady state concentration was $\approx 15–22$ d for lutein. The corresponding time observed for synthetic zeaxanthin was in the same range, $\approx 17$ d (22).

Peak steady state concentrations on day 42 were reached, on average, between 9 and 12 h after dosing; this finding agrees with the $t_{\text{max}}$ values observed for other compounds that enter the circulation via the lymphatics incorporated into chylomicrons and eventually get recirculated within hepatic VLDL (38). Mean dose-normalized $\Delta C_{\text{max}}$, all-E-lutein concentrations and $\Delta \text{AUC}$ values for group 2 were $\approx 64\%$ of those of group 1. Because the disposition parameter $k_e$ was not significantly different between the 2 lutein-supplemented groups, the observed nonlinearity was not related to dose-dependent disposition kinetics. Therefore, as discussed for other carotenoids, the capacity for intestinal absorption or recirculation within VLDLs may become limiting with increasing dose (39). For all-E-zeaxanthin, which was provided at much lower dosages, the plasma response appeared to be dose proportional, because dose-normalized $C_{\text{max}}$ and $\Delta \text{AUC}$
concentrations did not differ significantly. By contrast, at dosages of 1 and 10 mg zeaxanthin (22), nonlinearity was similar to that of lutein in the present trial.

The observation that the increases in plasma lutein concentrations were sigmoidally related to baseline concentrations (Figure 5) confirmed similar findings by Olmedilla et al (40). Several factors, including the disposition parameter \( k_p \), plasma cholesterol and triacylglycerol concentrations (as indexes of plasma lutein cotransport by lipoproteins), and sex were tested to account for the observed dependency in the present study. None of these parameters was significantly correlated with baseline concentrations or with the increase in \( \Delta C_{\text{me}} \) (data not shown). It appears that the variability in the response of plasma concentrations may be related to individuality in intestinal absorption or other metabolic events.

The half-life of apparent lutein disposition was comparable with effective half-life \( (t_{\text{eff}}) \), characterizing lutein accumulation during the approach to plateau. Such accumulation is characterized by an effective half-life, which is a weighted average of the absorption half-life and the half-lives describing the disappearance from plasma (41). The effective half-life is always shorter than the terminal half-life. The similarity of \( t_{\text{d}} \) and \( t_{\text{eff}} \) values indicates that \( t_{\text{d}} \) represents an estimate of the effective half-life rather than that of the terminal half-life. This is further corroborated by the finding that the constant term B, obtained from data fitting with the monoexponential plus constant term model, consistently exceeded the baseline lutein concentrations. The monoexponential plus constant term model does not represent the general integrated form of a corresponding compartmental model, and the prediction that plasma concentrations would not return to baseline concentrations could point to a biexponential model with a vanishing exponential term. Thus, a monoexponential plus constant term model may be interpreted as an approximation to the integrated form of a 2-compartment model (2 exponential terms) for the extreme case when the second rate constant approaches zero, ie, the terminal half-life becomes very long and may not be evaluated from data obtained within the selected time window. Such interpretation implies that a very slow plasma disappearance will become indistinguishable from a constant plasma concentration, particularly when the quality of the concentration data are corrupted by measurement errors. In fact, much longer lutein half-lives of \( \approx 15 \text{ d} \) (20) and 76 d (21) were previously published.

Furthermore, the mean \( t_{\text{d}} \) (5.5 d) was very similar to the \( t_{\text{eff}} \) (5.2 d) for zeaxanthin found in our previous study and was distinct from the reported terminal half-life of \( \approx 12 \text{ d} \) (22). Estimation of the terminal half-life of zeaxanthin in that study was critically dependent on the extended sampling interval to up to 76 d. An alternative explanation to account for the preference of the monoexponential plus constant term model might be the lack of adequate control of the dietary restrictions of lutein ingestion during the trial, which could have resulted in lutein intakes that surpassed prestudy intakes. However, this explanation appears to be highly unlikely on the basis of the subjects’ dietary records and on the basis of the finding that the constant term B exceeded baseline concentrations for all subjects. Moreover, the plasma lutein concentrations of the control subjects did not increase.

The accumulation index, which relates exposure at steady state to that after the first dose, is determined by the \( t_{\text{off}} \). The accumulation index was comparable for all-\( E \)-lutein (\( \approx 8.5 \) on average) and all-\( E \)-zeaxanthin (\( \approx 8 \), assuming that the \( t_{\text{d}} \) reflects \( t_{\text{eff}} \)), and there was excellent agreement with the accumulation index previously determined for zeaxanthin (\( \approx 7.5 \)) (Table 2) (22).

A considerable accumulation of plasma 3'-dehydro-lutein was observed in response to administration of the lutein preparation. Kinetics of 3'-dehydro-lutein resembled that of lutein, and we postulated that 3'-dehydro-lutein is formed from lutein. Substantiation of this hypothesis followed the same line of argument as described for formation of 3'-dehydro-lutein from zeaxanthin (22). Plasma 3'-dehydro-lutein concentrations were coupled to linearly interpolated all-\( E \)-lutein concentrations, which served as input function for driving the metabolite subsystem. This approach allowed for approximation of the kinetics of plasma 3'-dehydro-lutein, assuming first-order kinetics for formation and elimination of the compound, as described by a one-compartment model. The predicted concentrations were not significantly different from experimental plasma concentrations of 3'-dehydro-lutein (Figure 6). Furthermore, the increases in plasma lutein and 3'-dehydro-lutein at steady state were found to be proportional (Figure 7). These findings provide strong evidence that the rise in 3'-dehydro-lutein above baseline concentrations was closely related to plasma lutein concentrations and was, therefore, a consequence of lutein supplementation.

Because the lutein preparation also contained a small fraction of zeaxanthin, 3'-dehydro-lutein formation was probably in part derived from zeaxanthin (22). Therefore, the probable contribution of 3'-dehydro-lutein production from zeaxanthin to total metabolite was assessed by referring to the rate constants for formation of 3'-dehydro-lutein from zeaxanthin and eventual elimination (22) and by using the average linearly interpolated plasma all-\( E \)-zeaxanthin concentrations of group 2 (present

### TABLE 4

<table>
<thead>
<tr>
<th>Group</th>
<th>Lycopene</th>
<th>( \beta )-Carotene</th>
<th>( \alpha )-Carotene</th>
<th>( \beta )-Cryptoxanthin</th>
<th>Retinol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( C_B )</td>
<td>0.830 ± 0.471</td>
<td>0.510 ± 0.231</td>
<td>0.085 ± 0.048</td>
<td>0.337 ± 0.170</td>
<td>1.740 ± 0.403</td>
</tr>
<tr>
<td>( C_{SS} )</td>
<td>0.654 ± 0.226</td>
<td>0.719 ± 0.322</td>
<td>0.098 ± 0.071</td>
<td>0.290 ± 0.144</td>
<td>1.736 ± 0.387</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( C_B )</td>
<td>0.452 ± 0.223</td>
<td>0.503 ± 0.635</td>
<td>0.133 ± 0.189</td>
<td>0.155 ± 0.106^2</td>
<td>1.717 ± 0.284</td>
</tr>
<tr>
<td>( C_{SS} )</td>
<td>0.537 ± 0.336</td>
<td>0.493 ± 0.328</td>
<td>0.104 ± 0.080</td>
<td>0.196 ± 0.093</td>
<td>1.718 ± 0.303</td>
</tr>
</tbody>
</table>

\(^1\) All values are \( \bar{x} \) ± SD; \( n = 8 \) per group. Data for both sexes were pooled and represent the sum of \( E \) and \( Z \) isomers. \( C_B \) baseline concentration; \( C_{SS} \) time-averaged concentration calculated as area under the curve/24 on day 42. There were no significant differences between baseline and steady state concentrations.

\(^2\) Significantly different from group 1, \( P < 0.05 \).
study) as input function for driving the conversion to all-\(E\)-3'-dehydro-lutein. The simulation showed that \(\approx 18\%\) of the increase in 3'-dehydro-lutein concentrations may be related to the added zeaxanthin. The contribution at steady state was calculated from the \(\Delta\)AUC for all-\(E\)-zeaxanthin of the present study and from the slope and intercept of Figure 5 of reference 22. Again, \(\approx 18\%\) of the all-\(E\)-3'-dehydro-lutein was estimated to be formed from all-\(E\)-zeaxanthin. This confirms that lutein represented the major source of 3'-dehydro-lutein formation.

The model parameter characterizing the formation of 3'-dehydro-lutein, \(k_3\), is associated with a half-life of \(\approx 46\) d, which is much longer than the apparent half-life for lutein disposition (5.5–6 d). This implies that lutein is not exclusively eliminated via formation of 3'-dehydro-lutein, but also by other, more efficient, pathways, which may include metabolic reactions as suggested by Khachik et al (23). Furthermore, this is consistent with the hypothesis that lutein is also eliminated by a comparatively slow process (with a half-life of 46 d) as postulated above.

The 3'-dehydro-lutein formation rate constant from zeaxanthin (\(k_i = 0.055 \text{d}^{-1}\)) (22) significantly exceeded that from lutein (\(k_i = 0.017 \text{d}^{-1}\)) (Table 3). By contrast, the disposition constants of 3'-dehydro-lutein were not significantly different: \(k_i = 0.28 \text{d}^{-1}\) (22) and \(k_i = 0.24 \text{d}^{-1}\) (present study) when zeaxanthin or lutein were supplemented, respectively. Therefore, plasma disposition does not depend on the parent compound of the metabolite. The half-life associated with \(k_i\) is \(\approx 2.8\) d and, accordingly, the formation of 3'-dehydro-lutein was limited by the rate of formation.

In conclusion, the present study showed that plasma lutein concentrations increased 3.5-fold and 10-fold on average, respectively, after the long-term intake of 4.1 and 20.5 mg lutein. Plasma concentrations of all-\(E\)-3'-dehydro-lutein increased in parallel with those of all-\(E\)-lutein, and the increase was clearly related to lutein intake. Except for zeaxanthin, which was also present to a small portion in the preparation administered, other carotenoids remained unaffected by lutein administration.

PAT was responsible for the clinical conduct of the study, was involved in the design of the protocol (principal investigator according to GCP guidelines), and wrote the first draft of the manuscript. J-CA was responsible for the carotenoid and lipid analyses. WS initiated and supervised the project. UT was responsible for the clinical conduct of the study, was involved in the kinetic and statistical data analyses, and prepared the final manuscript. PAT was responsible for the clinical conduct of the study, was involved in the kinetic and statistical data analyses, and prepared the final manuscript. PAT received research grants for the conduct of the clinical part of the study from DSM Nutritional Products Ltd (formerly Roche Vitamins Ltd). J-CA, WS, and WC are employees of DSM Nutritional Products Ltd. UT had no conflict of interest.

REFERENCES


Long-term calcium supplementation does not affect the iron status of 12–14-y-old girls

Christian Mølgaard, Pernille Kæstel, and Kim F Michaelsen

ABSTRACT

Background: Single-meal studies have established that calcium has an acute inhibitory effect on the absorption of iron. However, there is growing evidence that high calcium intakes do not compromise iron status.

Objective: We evaluated whether long-term calcium supplementation taken with the main meal affected biomarkers of iron status in adolescent girls with high requirements of both iron and calcium.

Design: The study was a randomized, double-blind, placebo-controlled trial of supplementation with 500 mg Ca/d for 1 y among 113 adolescent girls aged 13.2 ± 0.4 y at enrollment. Participants were advised to take the supplement with their evening meal, which usually contributes the majority of dietary iron. Iron status was assessed at baseline and after 1 y of supplementation by measuring hemoglobin and serum concentrations of ferritin and transferrin receptors (TfRs).

Results: The mean (±SD) hemoglobin at enrollment was 134 ± 9 g/L, geometric mean serum ferritin was 26.3 µg/L (interquartile range: 18.6–39.4 µg/L), and serum TfR was 4.19 mg/L (3.52–5.10 mg/L). Daily calcium supplementation had no effect on the least-squares mean concentrations of iron-status markers adjusted for their baseline values (hemoglobin: 136 and 134 g/L, P = 0.73; ferritin: 25.4 and 26.1 µg/L, P = 0.12; and the ratio of TfR to ferritin: 160 and 161 in the calcium and placebo groups, respectively; P = 0.97).

Conclusion: Although it remains to be shown in iron-deficient persons, long-term iron status does not seem to be compromised by high calcium intakes.

KEY WORDS Iron status, intervention, calcium supplementation, calcium intake, adolescence, puberty, girls

INTRODUCTION

Calcium intake during the rapid growth in adolescence is considered important for primary prevention of osteoporosis, and calcium supplementation is often recommended to young girls with low dietary calcium intake (1). Also during adolescence, iron requirements increase because of rapid growth (increased skeletal muscle and blood volume); in females, moreover, they increase because of menstrual blood loss after menarche (2, 3). It has been shown in single-meal studies that calcium has an acute inhibitory effect on the absorption of both heme and nonheme iron (4–6).

It remains unclear whether whole-body retention of radiolabeled iron from complete diets is affected by calcium intake. Some studies suggest that calcium intake does not affect whole-body retention from complete diets for 4–5 d (7, 8), whereas consumption of dairy products with iron-rich meals during a 10-d period was shown to reduce iron absorption significantly (9). On the basis of the potentially negative influence of high calcium intake on iron absorption, it has been suggested that the intake of calcium (ie, milk or supplements) together with iron-rich meals should be avoided (1, 4). However, iron absorption data from single-meal or short-term studies may not be directly translated into the influence of long-term calcium supplementation on iron status. Indeed, long-term studies of calcium supplementation were conducted in different population groups and unequivocally showed no effect of long-term calcium supplementation on iron-status indicators (3, 6, 10–12). However, as far as we are informed, only one study has evaluated the effect on adolescent girls (3), a vulnerable group regarding iron status. We measured iron-status markers in 12–14-y-old girls participating in a randomized controlled study providing a daily dose of 500 mg calcium for a period of 1 y. As previously reported, calcium supplementation induced a modest increase in bone mineralization (13).

SUBJECTS AND METHODS

Subjects

All girls (n = 1213) with Danish names (both first names and surnames) aged 12 ± 0.5 y from Frederiksberg and Copenhagen municipalities were sent a food-frequency questionnaire (FFQ) for recording of dietary calcium intake. The FFQ included 88 food items from which usual daily intake (in mg/d) during the past month was estimated. The FFQ was previously validated against weighed food records (14). The girls were asked to complete the FFQ and return it together with information on their

1 From the Department of Human Nutrition, Centre for Advanced Food Studies, Royal Veterinary and Agricultural University, Frederiksberg, Denmark (CM, PK, and KFM).

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health, weight, and height. Six hundred eight girls (50%) returned the FFQ. Two groups were selected according to their dietary calcium intake: a group with intakes between the 40th and 60th centiles (1000–1304 mg/d; medium-intake group; n = 121) and a group with intakes below the 20th centile (<713 mg/d; low-intake group; n = 120). After exclusion because of nonwhite racial origin, abnormal weight for height (<3rd or > 97th centile (15)), diseases or intake of drugs with a potential effect on bones, 105 (medium-intake group) and 83 (low-intake group) were eligible for the present study. From these intake groups, 60 and 53 girls, respectively, agreed to participate.

Written informed consent was obtained from all of the participants and their parents. The study was approved (approval no. J nr (KF) 01–033/95) by the Ethics Committee for Copenhagen and Frederiksborg.

Design

The study was a randomized controlled trial designed to evaluate interactions between calcium supplementation and habitual calcium intake by stratifying the randomization according to habitual calcium intake. Girls from each of the 2 calcium intake groups were randomly assigned to receive either 500 mg Ca/d as CaCO₃ or placebo (microcrystalline cellulose) daily for 1 y. The girls were advised to take their supplement with their main (evening) meal to increase the compliance. The supplements were identical in appearance, and the intervention code was unknown to the study participants and investigators. At baseline and after 1 y, height and weight were measured, and dietary calcium intake was assessed by FFQ. In addition, blood was analyzed for concentrations of hemoglobin, serum ferritin, and transferrin receptors (TfRs). Compliance was evaluated by tablet count and expressed in percentage [(number of tablets eaten/number of tablets that should have been eaten) × 100].

Anthropometry, body composition, and pubertal stage

Height was measured to the nearest millimeter by using a wall-mounted stadiometer. While the subjects were wearing underclothes, weight was measured to the nearest 0.1 kg with an electronic digital scale. Pubertal development was evaluated as before or after menarche. Most of the girls underwent menarche before the end of the study period, and menarche at baseline was used to indicate the pubertal stage of the girls. Lean body mass (LBM) was measured by using dual-energy X-ray absorptiometry (DXA) with a Hologic 1000/W scanner (Hologic Inc, Waltham, MA) and by using HOLOGIC software (version 5.61; Hologic Inc). Details about the procedure were reported previously (13).

Laboratory methods

Hemoglobin was measured with a Sysmex analyzer (KX-21; Sysmex Corporation, Kobe, Japan). The cutoff for anemia was 110 g/L, according to Danish guidelines (16). The concentration of soluble TfRs was measured in duplicate by using an enzyme immunoassay kit (catalog no. TFX-94; Ramco Laboratories Inc, Houston, TX). Serum ferritin was measured in duplicate by using a fluoroimmunoassay kit (B069-101, DELFIA Ferritin; Wallac, Turku, Finland) that had a detection system based on a europium-labeled monoclonal antibody to human ferritin. Low iron status and iron deficiency were defined as ferritin <20 and <12 μg/L, respectively (17). The ratio of TfR to ferritin (both concentrations in μg/L) was calculated as a measure reflecting total body iron, with TfR:ferritin being negatively associated with body iron.

Statistical analysis

All statistical analyses were performed with SPSS software (version 12.0; SPSS Inc, Chicago). Normally distributed variables are given in means ± SDs, and variables that did not confine to normality are given in geometric means and interquartile ranges. Values of ferritin and TfRs were log₁₀ transformed to obtain normally distributed variables. Difference in iron-status markers were normally distributed and presented as mean differences and 95% CIs. Iron status after 1 y was compared between the 2 intervention groups by using analysis of covariance, in which the baseline value of the dependent variable was included as a covariate to adjust for baseline imbalances. P values < 0.05 were considered significant. Habitual calcium intake × intervention, low iron status × intervention, and change in LBM × intervention interactions were tested, and those with P values < 0.10 were considered significant. The prevalence of low iron status and iron deficiency after 1 y was compared between intervention groups by using logistic regression after adjustment for baseline prevalence.

RESULTS

Of the 113 girls who were included, 111 (98.2%) completed the trial. Median compliance during the intervention period was 86% (ie, 430 mg calcium/d), and there were no significant differences among the 4 groups (P = 0.44, one-way ANOVA). The mean (±SD) hemoglobin concentration at enrollment was 134 ± 9 g/L, the geometric mean (interquartile range) serum ferritin concentration was 26.3 (18.6–39.4) μg/L, and the geometric mean (interquartile range) TfR was 4.19 (3.52–5.10) mg/L. Depleted iron stores (ferritin <12 μg/L) were evident in 11 girls (9.8%), of whom 2 had TfR > 8.3 μg/L, which indicated tissue iron deficiency. According to the Danish cutoff for anemia in children aged 7 to ≤14 y (ie, 110 g/L; 16), none of the girls had anemia. At inclusion, the 65 girls (61%) who had already undergone menarche had significantly lower serum ferritin concentrations than did premenarcheal girls [geometric x: 23.8 (95% CI: 20.6, 27.4) μg/L among postmenarcheal girls and 30.7 (25.5, 36.9 μg/L among premenarcheal girls; P = 0.029). Correspondingly, girls who had undergone menarche tended to have significantly higher TfRs than did premenarcheal girls [geometric x: 4.37 (95% CI: 4.07, 4.68) mg/L and 3.89 (3.47, 4.37) mg/L, respectively; P = 0.10]. However, the risk of iron deficiency (ferritin <12 μg/L) was not related to menarche status [10.4% in postmenarcheal girls and 8.9% in premenarcheal girls; P = 0.79 (Pearson’s coefficient)].

Body size, age, and menarcheal status were well balanced between intervention groups at baseline (Table 1). Unfortunately, serum ferritin was significantly lower in the placebo group. Accordingly, baseline values were included in all analyses of iron status at follow-up to adjust for this imbalance. Iron status did not differ according to habitual calcium intake, and the effect of calcium supplementation did not depend on habitual calcium intake (no significant interactions, Table 2).

Least-squares means (LSMs) adjusted for baseline values did not differ between the 2 intervention groups for any of the iron-status
markers. In other words, there was no effect of calcium supplementation on either of the evaluated iron-status markers after 1 y of calcium supplementation.

Furthermore, calcium supplementation did not significantly affect the proportion of girls having low iron status (odds ratio (OR) for ferritin $\geq 20$ μg/L; 95% CI: 0.94, 2.73) or iron deficiency (OR $\leq 12$ μg/L; 0.58; 95% CI: 0.12, 2.85) after adjustment for baseline iron deficiency.

To explore whether the results were likely to be biased by the skewed distribution of iron status, subjects were stratified according to whether they had replete or low iron status at baseline (the cutoff was 20 μg ferritin/L). The effect of calcium supplementation did not appear to depend on initial iron status according to the estimates in the different strata (data not shown).

LBM expansion during growth may negatively affect the iron stores of adolescent girls, as shown in a study with a 4-y follow-up (3). In our study, the mean absolute change in LBM during 1 y was 1.24 kg, which corresponds to a monthly gain of 103 g LBM. There was no association between serum ferritin and the 1-y change in LBM either with or without adjustment for baseline ferritin (unadjusted estimate for log ferritin: $B = 0.004$; 95% CI: $0.023, 0.016$; $P = 0.71$). Similarly, there

<table>
<thead>
<tr>
<th>Characteristic and calcium intake</th>
<th>Placebo group</th>
<th>Calcium group</th>
<th>Intervention</th>
<th>Habitual calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y) Low Ca</td>
<td>13.2 ± 0.4</td>
<td>13.3 ± 0.3</td>
<td>0.68</td>
<td>0.41</td>
</tr>
<tr>
<td>Age (y) Medium Ca</td>
<td>13.2 ± 0.4</td>
<td>13.1 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (cm) Low Ca</td>
<td>159.6 ± 7.8</td>
<td>159.6 ± 6.5</td>
<td>0.76</td>
<td>0.04</td>
</tr>
<tr>
<td>Height (cm) Medium Ca</td>
<td>161.9 ± 6.3</td>
<td>163.5 ± 6.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg) Low Ca</td>
<td>49.2 ± 8.7</td>
<td>52.3 ± 8.5</td>
<td>0.23</td>
<td>0.74</td>
</tr>
<tr>
<td>Weight (kg) Medium Ca</td>
<td>50.7 ± 9.0</td>
<td>51.8 ± 9.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/L) Low Ca</td>
<td>134 ± 8</td>
<td>134 ± 9</td>
<td>0.89</td>
<td>0.92</td>
</tr>
<tr>
<td>Hemoglobin (g/L) Medium Ca</td>
<td>134 ± 10</td>
<td>134 ± 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferritin (g/L) Low Ca</td>
<td>23.4 (17.6–34.6)</td>
<td>27.5 (20.1–37.5)</td>
<td>0.009</td>
<td>0.51</td>
</tr>
<tr>
<td>Ferritin (g/L) Medium Ca</td>
<td>22.4 (15.6–36.3)</td>
<td>33.9 (22.6–44.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TfR (mg/L) Low Ca</td>
<td>4.3 (3.6–5.1)</td>
<td>4.1 (3.5–4.9)</td>
<td>0.59</td>
<td>0.92</td>
</tr>
<tr>
<td>TfR (mg/L) Medium Ca</td>
<td>4.3 (3.5–5.1)</td>
<td>4.3 (3.2–5.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TfR:ferritin Low Ca</td>
<td>186 (94–279)</td>
<td>145 (89–213)</td>
<td>0.04</td>
<td>0.48</td>
</tr>
<tr>
<td>TfR:ferritin Medium Ca</td>
<td>191 (90–286)</td>
<td>123 (85–190)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

1. TfR, transferrin receptor. Low calcium intake, <713 mg Ca/d ($n = 27$ and 30 for placebo and calcium groups, respectively); medium calcium intake, 1000–1304 mg Ca/d ($n = 26$ and 30 for placebo and calcium groups, respectively).
2. t Test for the main effect of calcium supplementation versus placebo.
3. t Test for the main effect of low versus medium habitual calcium intake.
4. $x \pm SD$ (all such values).
5. Geometric $x$; interquartile range in parentheses (all such values).

---

Table 2

Iron-status markers after 1 y of calcium supplementation among 111 young girls according to intervention group and habitual calcium intake

<table>
<thead>
<tr>
<th>Placebo group</th>
<th>Calcium group</th>
<th>Main effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/L) Low Ca ($n = 27$)</td>
<td>134 (131, 137)</td>
<td>135 (132, 137)</td>
</tr>
<tr>
<td>Hemoglobin (g/L) Medium Ca ($n = 24$)</td>
<td>135 (132, 137)</td>
<td>135 (132, 138)</td>
</tr>
<tr>
<td>Ferritin (g/L) Low Ca ($n = 27$)</td>
<td>26.9 (22.6, 31.9)</td>
<td>24.9 (20.9, 29.7)</td>
</tr>
<tr>
<td>Ferritin (g/L) Medium Ca ($n = 24$)</td>
<td>24.9 (20.9, 29.7)</td>
<td>24.3 (20.3, 29.2)</td>
</tr>
<tr>
<td>TfR (mg/L) Low Ca ($n = 27$)</td>
<td>4.1 (3.8, 4.5)</td>
<td>4.5 (4.2, 4.9)</td>
</tr>
<tr>
<td>TfR (mg/L) Medium Ca ($n = 24$)</td>
<td>4.5 (4.2, 4.9)</td>
<td>4.0 (3.7, 4.4)</td>
</tr>
<tr>
<td>TfR:ferritin Low Ca ($n = 27$)</td>
<td>145 (123, 171)</td>
<td>177 (145, 217)</td>
</tr>
<tr>
<td>TfR:ferritin Medium Ca ($n = 24$)</td>
<td>177 (145, 217)</td>
<td>163 (137, 194)</td>
</tr>
</tbody>
</table>

1. TfR, transferrin receptor. Two of the 113 girls studied did not complete the trial.
2. Analysis of covariance. Habitual, low or medium calcium intake; Supplemental, calcium supplementation or placebo.
3. Habitual x Supplemental.
4. Least-squares $\bar{x}$; 95% CI in parentheses (analysis of covariance with baseline values of the dependent variable included as covariate).
5. Geometric $\bar{x}$ of the least-squares $\bar{x}$ (analysis of covariance with baseline values of the dependent variable included as covariate).
were no effects of the change in LBM on TfR (unadjusted estimate for log TfR: B = −0.004; 95% CI: −0.017, 0.010; P = 0.62), and nor was change in LBM an effect modifier of supplementation for any of the iron-status markers (LBM × intervention group interaction for hemoglobin, ferritin, and TfR, P > 0.10).

DISCUSSION

The long-term effect of daily calcium supplementation on iron status was investigated in a group of adolescent girls around the time of menarche, when the requirements of iron and calcium are high. Daily calcium supplementation for 1 y had no effect on iron stores assessed by serum ferritin, no effect on tissue iron status assessed by TfR, and no effect on TfR:ferritin, which is closely associated with total body iron estimated from several iron-status markers (18, 19). Moreover, hemoglobin was unaffected by calcium supplementation.

Even though large sample sizes are generally needed to verify negative findings, we believe that it is unlikely that the lack of effect was due to limited power, because the LSM suggested very small differences, if any. Thus, we do not completely reject the hypothesis that calcium supplementation impairs iron status, but our data suggest that any effects are negligible, as indicated by the very small differences in the LSMS. Furthermore, the estimates for the different iron-status markers point in different directions, which supports the argument that the effects are random errors around unity.

The girls were in the age around the time of menarche, when iron stores begin to be used to compensate for menstrual losses. At baseline, menarcheal status was significantly associated with iron status but not with iron deficiency. Furthermore, circulating TfRs tended to be higher, although not significantly (P = 0.10), which indicated a lower tissue iron availability among girls who had undergone menarche.

The study participants were advised to take the supplement with their main (evening) meal, which usually contributes the highest amount of both heme and nonheme iron. Thus, the girls were not advised to separate the calcium supplements from the iron-rich meals as has been recommended (4), and our study illustrates a high iron absorption–inhibiting potential. However, this potential may have been further amplified by supplementing at 2 or 3 meals daily. The inhibiting effect of calcium on iron absorption seen in single-meal studies appears to be dose related in the range from 40 to 300 mg, and there is no further effect at higher doses (20). Therefore, a higher iron-inhibiting potential could theoretically be obtained by distributing the total amount of 500 mg/d across more of the daily meals.

The main concern of our study is whether the estimates are biased by the baseline imbalance in iron status between the intervention groups. If baseline iron status does not interact with the effect of calcium supplementation on iron status, then control for initial values in the analyses will provide valid estimates. If however, the effect of calcium supplementation is influenced by iron status, then our results may be biased.

It is well known that iron status is the main determinant of the efficiency of iron absorption and that increasing absorption is a consequence of decreasing iron status (6, 21). Thus, there is reason to believe that the effect of modifiers of iron bioavailability is more pronounced with decreasing iron status. If this is the case, then the effect of calcium supplementation could be underestimated in our study because iron status was lower in the placebo group than in the calcium group. Because of this imbalance, the study had little power to assess the interaction between iron status and calcium supplementation. On the other hand, restriction of the analysis to subjects with low iron status at baseline did not show a negative influence of calcium supplementation on iron status. In conclusion, we have no reason to doubt the validity of our estimates.

The long-term effect of calcium on iron status was previously investigated in different population groups, including healthy adults of both sexes (6), lactating and nonlactating women (10, 22), and adolescent females (3). None of those studies found any evidence that calcium supplementation compromised long-term iron status assessed by serum ferritin. Similarly, there was no difference in the incorporation of iron in red blood cells between preschool children who consumed low-calcium diets for 5 wk and those who consumed high-calcium diets (12).

None of the above studies used the TfR as an indicator of iron status. However, particularly in populations with low iron stores, it may be useful, in addition to serum ferritin, because the concentration of TfR in serum increases with decreasing iron status beyond the point of depleted iron stores (serum ferritin <12 μg/L), whereas serum ferritin is not very sensitive to further depletion of body iron stores (17, 23). One of the above studies was conducted among Gambian lactating women with a high prevalence of iron deficiency (≈40% had <12 μg serum ferritin/L; 22). Unfortunately, hemoglobin and serum ferritin were the only iron-status markers used in that study. In the current study, we performed further evaluations of persons with low iron stores (<20 μg/L), among whom the effect would probably be most pronounced. This approach did not suggest that there was a differential effect, for either ferritin or TfRs, among susceptible individuals with low iron stores.

We evaluated whether there was an association between change in LBM and iron status assessed by serum ferritin and TfR. An inverse relation between an increase in LBM and iron status would be plausible because iron stores are used to cover LBM requirements in periods with increased needs due to, for example, construction of muscle tissue. This has been shown in a group of American girls aged 11–y (3). The mean increase in LBM in the current study was only 1.2 kg/y, whereas it was 2.9 kg/y in the American study. Age at enrollment was 13.2 y in the current study and 10.8 y in the American study, and the reason that we did not observe the association may be that most of the girls in our study were old enough to have passed the time for maximum gain of LBM.

The lack of effect on long-term iron status of calcium supplementation, despite a well-established inhibiting effect of iron absorption observed in single-meal studies, may represent a phenomenon that is not specific to the iron × calcium interaction. For example, the enhancing effect of ascorbic acid on iron absorption does not appear to extrapolate to an effect on iron status of long-term high intakes of ascorbic acid (24). Short-term studies of mineral metabolism probably are primarily of mechanistic interest, and public health recommendations should always be based on long-term studies.

In conclusion, this study conducted among adolescent females during a period with high requirements of both iron and calcium did not suggest any adverse effects on iron status of daily calcium supplementation with 500 mg Ca in addition to a relatively high
dietary calcium intake. However, before the long-term effects of calcium supplementation on iron status can be fully rejected, it remains to be shown that there is no effect in iron-deficient persons (11).

We are grateful to Birgitte Hermansen for carrying out most of the practical work in the study.

CM and KFM designed the study. CM was responsible for the collection of data. PK was responsible for data analysis and prepared the first draft manuscript. All authors participated in the discussion of results, commented on the manuscript, and approved the final manuscript. None of the authors had any financial or personal conflicts of interests.

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Long-term moderate zinc supplementation increases exchangeable zinc pool masses in late-middle-aged men: the Zenith Study

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ABSTRACT

Background: Zinc supplementation may be beneficial for health. Assessing exchangeable zinc pools may be a useful approach to evaluate zinc status.

Objective: We evaluated the effects of long-term supplementation with 2 moderate doses of zinc on the mass of exchangeable zinc pools.

Design: Three groups of healthy, late-middle-aged men (n = 16 per group) participated in a stable-isotope zinc kinetic study after 6 mo of daily supplementation with 0 (placebo), 15, or 30 mg Zn. At the end of the supplementation period, each subject received an intravenous injection of 0.89 mg 70Zn, and the plasma zinc disappearance curve was monitored for the next 10 d. Two approaches were used to determine the characteristics of the exchangeable zinc pools: 1) formal 3-compartmental modeling and 2) a simplified determination of the total mass of the rapidly exchangeable zinc pool (EZP).

Results: In the placebo group, the exchangeable zinc pool masses for the 3 considered pools were as follows: 2.15, 12.7, and 100.5 mg Zn. The rapidly exchangeable zinc pool mass in the placebo group was 143 mg Zn. Zinc supplementation significantly increased the exchangeable zinc pool masses regardless of the approach used to determine these pools. In addition, these data confirm that exchangeable zinc pool masses correlate positively with total zinc intake and negatively with subject age and do not correlate with plasma zinc concentrations.

Conclusion: Our data show that long-term supplementation with 2 moderate doses of zinc is an efficient way to increase exchangeable zinc pool masses in late-middle-aged men. Am J Clin Nutr 2005;82:103–10.

KEY WORDS Exchangeable zinc pools, kinetic modeling, stable isotope, zinc supplementation, late-middle-aged subjects, zinc status

INTRODUCTION

Zinc plays an important role in nutrition and health (1). It is involved in many enzymatic reactions, and several hormones are also known to be zinc-dependent (2). Thus, zinc plays an essential role in a wide range of fundamental cellular reactions, and zinc deficiency may be implicated in various metabolic disorders. In developed countries, marginal zinc intake may induce a high prevalence of marginal zinc deficiency, which in turn may contribute to various chronic and degenerative diseases associated with aging (1). Inadequate zinc intakes have been shown in elderly adults (3). Zinc supplementation may thus be beneficial for health, notably in late-middle-aged populations. This age bracket represents an important biological age for understanding the mechanisms of pre-aging before the appearance of aging-related diseases.

Sensitive and specific markers of zinc status are needed to achieve the most reliable estimates of zinc requirements. The approach most often used for assessing zinc status is the measurement of plasma zinc concentrations. However, many factors that are not directly related to zinc nutriture affect this measurement (4). Other static measures, such as urinary zinc excretion or zinc content in blood cells, can be performed, but they are not always reliable indicators of zinc status (4). Functional indicators may also be measured, such as plasma alkaline phosphatase activity or other zinc metalloenzyme activities in tissues; however, they have some limitations (4). The study of exchangeable zinc pools with the use of a stable isotope has been developed as a useful approach for evaluating zinc status (5). However, although numerous studies have explored the effect of zinc depletion alone or the effect of zinc depletion and repletion in humans (6–11), few data, if any, are available in the literature on the effect of moderate doses of zinc supplementation on these zinc kinetic parameters.

Thus, the aim of this study was to explore the effect of long-term supplementation with 2 moderate doses of zinc on exchangeable zinc pool characteristics in late-middle-aged subjects. We compared the effects of a placebo with those of two doses of supplemental zinc (15 and 30 mg/d), which correspond to 1.5 and 3 times the recommended dietary allowances of zinc for the present study population.

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__2__The Zenith Study is supported by the European Commission “Quality of Life and Management of Living Resources” Fifth Framework Program, contract no. QLK1-CT-2001-00168.

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SUBJECTS AND METHODS

Subjects
Forty-eight healthy men aged 58–68 y participated in this study. The clinical examination was carried out by the medical staff at the Unit of Nutritional Exploration of the Human Nutrition Research Center in Clermont-Ferrand, France. All subjects were nonsmokers and judged to be healthy on the basis of a routine blood screening, a medical history, a physical examination, and a psychological profile. At the time of recruitment and throughout the duration of the study, none of the selected subjects took vitamin or mineral supplements or medications that may have affected mineral metabolism. Each subject’s body weight (in kg) and standing height (in cm) were recorded while they were wearing light indoor clothing and no shoes. Body mass index (in kg/m²) was calculated to screen for obesity. Skinfold thickness was assessed by using a Harpenden caliper, according to the procedure described by Durnin and Womersley (12). Skinfold thicknesses were measured at the biceps, triceps, subscapular, and suprailiac regions. The mean of 3 measurements per site was used in subsequent calculations. Fat-free mass was estimated from skinfold thickness with the use of Durnin and Womersley equations (12). Dietary zinc intake was estimated with use of 4-d food-intake records (which included weekend days) and the GENI program (MICRO 6; Villiers les Nancy, France). The subjects were asked to maintain their habitual diet and exercise patterns for the duration of the study. The study was approved by the local Human Ethical Committee in Clermont-Ferrand under number AU 478 (CCPPRB Auvergne). All subjects were fully informed of the purposes of the study and gave their written informed consent.

Experimental design
The study was a randomized, double-blind, placebo-controlled intervention trial conducted in late-middle-aged men. Each group contained 16 subjects, who were randomly assigned to receive placebo, 15 mg Zn/d, or 30 mg Zn/d for 6 mo. The supplemental zinc was given as zinc gluconate, which was prepared and supplied by E-Pharma (Creapharm, Gannat, France). The placebo capsule contained 199 mg lactose and 1 mg magnesium stearate. The 7.5-mg Zn capsule contained 56.9 mg Zn gluconate, 142.1 mg lactose, and 1 mg magnesium stearate. The 15-mg Zn capsule contained 113.7 mg Zn gluconate, 85.3 mg lactose, and 1 mg magnesium stearate. Zinc capsules were distributed to the subjects at the beginning of the trial and at 3 mo. At 3 and 6 mo, the subjects were asked to return any remaining capsules, and the degree of apparent compliance was estimated from the number of delivered capsules and the number of returned capsules. Compliance, expressed as a proportion of the intended supplements consumed during zinc supplementation, did not differ among the groups; the mean compliance was >98% in all groups. Zinc kinetic studies, with use of the 70Zn stable isotope, were performed in all subjects after 6 mo of supplementation (see below).

Preparation of stable-isotope solution
Zinc has 5 naturally occurring stable isotopes. The least naturally abundant stable isotope, 70Zn (natural abundance 0.62%), was used in this study. This isotope was purchased in the oxide form, and it had an enrichment of 95.4% (Chemgas, Paris, France). Bottles of the 70Zn stable-isotope solution were prepared by the hospital pharmacist (Clermont-Ferrand University Hospital Center, France). Briefly, 153.7 mg ZnO (125 mg Zn corresponding to 119.3 mg 70Zn) were first moistened with 2 mL milliQ water (MilliPore, Saint-Quentin en Yvelines, France), and then 1 mL concentrated HCl (12 mol/L) was added gradually with stirring to completely transform the oxide form into the chloride form. The solution volume was then adjusted to 200 mL with water containing 0.9% NaCl. This solution was gradually neutralized with 6 mL of 1 mol NaOH/L, to reach a pH between 4 and 7. The volume of this solution was then adjusted to 2000 mL with water containing 0.9% NaCl. The pH and osmolarity (270–320 mOsmol) were then checked. The solution was finally filtered on a 0.22-μm filter and divided into 130 vials (each containing 15-mL aliquots) that were then autoclaved for 20 min at 121 °C. The pharmaceutical quality of the stable-isotope solution (nontoxicity, sterility, and pyrogenicity) was certified by the Clermont-Ferrand Hospital pharmacy. The zinc concentration and the 70Zn isotopic enrichment of the prepared solutions were determined by inductively coupled plasma mass spectrometry (ICP-MS DRC6100; Perkin-Elmer, Paris, France) in our laboratory before use.

Tracer administration at the end of zinc supplementation
Six months after zinc supplementation, the subjects were admitted to the Human Nutrition Unit of the Clermont-Ferrand Hospital after an overnight fast. An intravenous catheter was inserted into the left arm of each subject, and ≈1 mg (0.89 ± 0.02 mg) 70Zn in 15 mL of saline solution was perfused over 2 min. (The end of perfusion was considered to be 0 h.) Another intravenous catheter was also inserted into the left arm of each subject for blood sampling at −0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 10 h. A blood sample, which was taken after the subjects had fasted for 12 h, was also obtained on days 1, 2, 3, 5, 7, and 10. A urine sample was collected from fasting subjects in the morning of the day before the isotope was administered.

Analysis
Blood samples (2 mL) were collected in zinc-free heparin-coated tubes, and a blood aliquot was used for the measurement of hematocrit. The blood sample was centrifuged (1000 x g, 15 min, 4 °C), and the plasma was separated. Erythrocytes that were collected 15 min before the start of the isotope study were washed with saline solution and hemolyzed. Urine samples were acidified with pure hydrochloric acid (final acid concentration: 1%). The samples were then stored at −20 °C until analyzed.

The 70Zn content in plasma samples was determined by ICP-MS (Perkin-Elmer). Before analysis, the plasma was diluted in 0.014 mol HNO3/L, so that total zinc concentrations in the analyzed samples were ≈100 μg/L. Natural zinc and indium (SPEX Claritas; SpexCertiPrep Inc, Metuchen, NJ) were used as external and internal standards, respectively. All isotope analyses were carried out at least twice. For the 70Zn/66Zn measurement, the within-day and between-day relative variations were 0.62% and 1.11%, respectively. The limit of detection for 70Zn/66Zn enrichment was ≈1.5%.

Total zinc concentrations were measured in plasma and urine samples (diluted in 1% HCl) with a flame atomic absorption spectrophotometer (AA800; Perkin-Elmer) at 214 nm. Urinary creatinine concentrations were measured by colorimetry. The
standard method used at the hematologic laboratory of Clermont-Ferrand Hospital was used to measure hematocrit in blood samples.

**Determination of exchangeable zinc pools**

First approach: kinetic analysis

Zinc kinetics were determined with the use of a multicompartamental model as described by Pinna et al (7), which, in turn, is based on a model developed by Wastney et al (13). A schema of the proposed model is shown in Figure 1. Compartmental modeling of the data was performed with the aid of the SAAM II (stimulation, analysis, and modeling) software package (SAAM Institute Inc, Seattle, WA; 14). Plasma stable-isotope data were expressed as tracer/tracee, with use of the following formula as described by Lowe et al (15):

\[
\text{Tracer/Tracee} = \left[ (m70/m66) + 0.0003(m67/m66) \right] - 0.0224 \left[ 3.0705 - 0.1358(m70/m66) - 0.0876(m67/m66) \right]
\]

where m70/m66 and m67/m66 are the isotopic ratios of \(^{70}\text{Zn}/^{66}\text{Zn}\) and \(^{67}\text{Zn}/^{66}\text{Zn}\), respectively, in the measured sample. The isotopic ratios m70/m66 and m67/m66 were determined by ICP-MS with the use of an external calibration curve of natural zinc.

The mass of the first exchangeable zinc pool (M1) was calculated on the basis of the plasma zinc concentration and the plasma volume and was fixed for computer fitting (16). Plasma volume was estimated in relation to the body weight of the subject with blood volume equal to 70 mL/kg body weight (17) and hematocrit values. The mass of the other exchangeable zinc pools (M2 and M3), the fractional transport rate [ie, the fraction leaving a compartment per unit time (exchange constant between pools \(k_{1,2}, k_{2,1}, k_{1,3},\) and \(k_{3,1}\))] and the irreversible loss of zinc from pool 1 (\(k_{0,1}\)) were determined from the model with use of SAAM II. The other kinetic parameters were determined as follows: flux of isotope \(k_{i,j}\) \(\times M_j\) (\(i\) and \(j\) are the pools being measured); plasma fractional turnover = \(k_{2,1} + k_{3,1} + k_{0,1}\); and plasma flux = plasma fractional turnover \(\times M1\). The half-lives of the 3 exchangeable zinc pools were determined from a 3-exponential curve with the use of SAAM II. The calculations of pool masses and half-lives were performed as previously described for magnesium pools (18).

Second approach: exchangeable zinc pool

We calculated the exchangeable zinc pool (EZP) mass according to the method described by Miller et al (11). This method assumes that \(^{70}\text{Zn}\) isotopes exchange with total plasma zinc at a rate that is fast enough to completely intermix within a 48-h period. The EZP is thus equivalent to the mass of the isotope administered divided by the tracer/tracee value at the y intercept of the linear regression of a semilogarithmic plot of the plasma tracer/tracee data between day 3 and day 10 (Figure 2). The rate constant \(k\) of the total flow of zinc out of the EZP is equal to the negative slope of this linear regression. The half-life of the EZP was calculated as previously described (18). The turnover time of the EZP was \(1/k\), and the flow rate out of the EZP was calculated by multiplying \(k\) with the EZP mass (9).

**Statistical analyses**

The results are expressed as means ± SDs. For exchangeable zinc pool characteristics, the statistical analyses were based on a one-factor analysis of variance followed by a Tukey-Kramer multiple comparisons test, or on a Kruskal-Wallis test on simple linear regression, and on Spearman’s correlation coefficients. The limit of statistical significance was set at \(P < 0.05\). For zinc status results, a 2-factor repeated measures analysis of variance with interactions was performed. Statistical analyses were performed with the use of GRAPHPAD software (version 3.00; GraphPad Software, San Diego, CA) and STATVIEW program (SAS Institute Inc, Cary, NC).

**RESULTS**

Subject characteristics and zinc nutriture at baseline

Age, body mass index, fat-free mass (FFM), and mean zinc intakes and plasma zinc concentrations were not significantly different between groups at baseline (Table 1). In the kinetic study, all subjects tolerated the intravenous \(^{70}\text{Zn}\) infusion well.

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**FIGURE 1.** Three-compartmental model of zinc kinetics. Adapted with permission from reference 7. Arrows represent intercompartmental movements of the cation determined by appropriate rate constants and irreversible loss.

**FIGURE 2.** Method used to calculate exchangeable zinc pools (EZPs) according to Miller et al (11). EZP mass is equivalent to the mass of the isotope administered divided by the tracer/tracee value at the y intercept of the linear regression of a semilogarithmic plot of the plasma tracer/tracee data between day 3 and day 10. Data presented are a typical example.
Effect of zinc supplementation on blood zinc concentrations and urinary zinc excretion

Plasma zinc concentrations increased significantly over the 6 mo of supplementation in the groups that received 15 and 30 mg Zn/d; no significant changes were observed in the placebo group (Table 1). Furthermore, plasma zinc concentrations increased significantly with zinc supplementation when the 3 groups of subjects were compared (Table 1). Baseline plasma zinc concentrations and urinary zinc excretion were not significantly different between groups (Table 1).

Effect of zinc supplementation on exchangeable zinc pools according to compartmental modeling

Data for a representative subject after intravenous $^{70}$Zn infusion are shown in Figure 3. The curve reflects the response generated by the model shown in Figure 1. The pattern of the curve showed a rapid disappearance of the tracer during the first 8 h, which was followed by a slow decline that extended through $>240$ h. The kinetic patterns were significantly different between the zinc-supplemented and -nonsupplemented groups (data not shown). The kinetic indexes, the masses of the exchangeable zinc pools, and the half-lives of these pools are listed in Table 2. Pool 1 is composed of plasma zinc, pool 2 is a fast pool composed of zinc found mainly in erythrocytes but also in parts of the liver, and pool 3 is a slow pool composed of zinc found in the liver and in bone (13).

$M_1$ was significantly increased with zinc supplementation (Table 2). $M_2$, $M_3$, and the sum of the 3 pools ($tM$) tended to increase with zinc supplementation ($P < 0.1$ for $tM$), but this was not statistically significant because of the large intravariability in the group who received the highest zinc dose (Table 2). Moreover, $M_1$, $M_2$, and $tM$ correlated significantly and $M_3$ only tended to be correlated ($P = 0.078$) with total dietary zinc intake (diet + supplement; Table 3). When these pool masses were adjusted for FFM, $M_1$/FFM and $tM$/FFM increased significantly with zinc supplementation ($P < 0.01$ and $P < 0.05$, respectively), whereas $M_2$/FFM and $M_3$/FFM only tended to increase ($P < 0.1$; Table 2). Surprisingly, $M_1$/FFM, $M_2$/FFM, and $M_3$/FFM were not correlated with plasma zinc concentrations and only EZP/FFM showed a trend toward a correlation ($r = 0.241$, $P = 0.0993$; $n = 48$; Table 3).

Fractional transport rates of zinc between pools, irreversible loss from pool 1, $\text{flux}_{1/FFM}$, and $\text{flux}_{3/FFM}$, and plasma turnover were not significantly different between the 3 subject groups. Plasma zinc flux and $\text{flux}_{2/1}$ significantly increased with zinc supplementation (Table 2) and were positively correlated with zinc intake (Table 3). The irreversible loss of zinc from pool 1 [$\text{flux}_{0/1}$] was negatively correlated with zinc intake ($P < 0.05$; Table 3). In addition, the half-lives of the pools were not significantly different between the 3 groups (Table 2). Finally, plasma zinc flux correlated with plasma zinc concentrations ($r = 0.474$, $P < 0.001$; $n = 47$; Table 3).

Effect of zinc supplementation on the exchangeable zinc pools according to the Miller model

The EZP and the EZP adjusted for FFM increased significantly with zinc supplementation (Table 4). Moreover, the EZP correlated significantly with total dietary zinc intake (diet + supplement; Table 3) and tended to correlate with plasma zinc concentrations ($r = 0.236$, $P = 0.1067$; $n = 48$). In addition, the EZP correlated strongly with $M_3$ ($r = 0.591$, $P < 0.001$), $tM$ ($r = 0.751$, $P < 0.001$; $n = 48$), and almost with $M_2$ ($r = 0.282$, $P = 0.0547$; $n = 48$). The half-life and turnover time of the EZP were not significantly different between the 3 groups of subjects. Nevertheless, the EZP turnover tended to correlate with zinc intake ($r = 0.259$, $P = 0.076$; $n = 48$). In addition, the flow-out rate was

---

**Table 1**

Characteristics of the subjects and zinc status at baseline and after 6 mo of zinc supplementation

<table>
<thead>
<tr>
<th></th>
<th>Placebo group</th>
<th>15-mg Zn group</th>
<th>30-mg Zn group</th>
<th>Effect of period</th>
<th>Effect of group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>62.8 ± 3.4</td>
<td>59.9 ± 4.0</td>
<td>61.3 ± 4.6</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>26.2 ± 2.3</td>
<td>26.2 ± 2.4</td>
<td>25.7 ± 2.6</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Fat-free mass (kg)</td>
<td>57.2 ± 5.2</td>
<td>56.8 ± 4.9</td>
<td>56.5 ± 3.8</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Habitual zinc intake (mg/d)</td>
<td>6.18 ± 2.02</td>
<td>7.23 ± 2.16</td>
<td>6.65 ± 1.62</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma zinc (μmol/L)</td>
<td>12.84 ± 1.69</td>
<td>14.18 ± 1.75</td>
<td>15.03 ± 2.24</td>
<td>0.018 $&lt;0.001$</td>
<td>0.030</td>
</tr>
<tr>
<td>RBC zinc (μmol/L)</td>
<td>247 ± 64</td>
<td>251 ± 51</td>
<td>227 ± 52</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Urinary zinc (μmol/mmol creatinine)</td>
<td>0.65 ± 0.25</td>
<td>0.77 ± 0.39</td>
<td>0.77 ± 0.25</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

1 All values are $\bar{x} ± SD$; $n = 16$. RBC, red blood cell.

2 Statistical significance was set at $P < 0.05$ (2-factor repeated-measures ANOVA).
not significantly different. However, the EZP correlated significantly with the half-life of $^{70}\text{Zn} (r = 0.660, P < 0.001; n = 48)$ and with turnover time ($r = 0.658, P < 0.001; n = 48$).

**Effect of age on the exchangeable zinc pool masses**

Both $tM$ and EZP correlated negatively with age in the nonsupplemented group (for $tM$: $r = -0.584, P < 0.05; n = 16$; for EZP: $r = -0.518, P < 0.05; n = 16$), and $M3$ tended to correlate with age ($r = -0.473, P = 0.064; n = 16$). When expressed in FFM, this correlation was attenuated; $tM/FFM$ was correlated with age ($r = -0.575, P < 0.05; n = 16$) but this was not the case for EZP/FFM or $M3/FFM$. Moreover, plasma turnover was correlated with age in the nonsupplemented group ($r = 0.504, P < 0.05; n = 16$). Zinc supplementation improved exchangeable zinc pool masses in the supplemented subjects, which canceled out the negative correlation between the exchangeable zinc pool masses and age that was observed in the placebo group.

**DISCUSSION**

In the present study, we examined the effect of long-term supplementation with 2 moderate doses of zinc (15 and 30 mg/d)

**TABLE 3**

Linear regression coefficients between exchangeable zinc pool masses ($M$) and zinc intake and plasma zinc concentrations

<table>
<thead>
<tr>
<th>Zinc intake</th>
<th>Plasma zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r$</td>
<td>$P$</td>
</tr>
<tr>
<td>$M1$</td>
<td>0.362</td>
</tr>
<tr>
<td>$M2$</td>
<td>0.289</td>
</tr>
<tr>
<td>$M3$</td>
<td>0.260</td>
</tr>
<tr>
<td>$tM$</td>
<td>0.398</td>
</tr>
<tr>
<td>$M1/FFM$</td>
<td>0.424</td>
</tr>
<tr>
<td>$M2/FFM$</td>
<td>0.311</td>
</tr>
<tr>
<td>$M3/FFM$</td>
<td>0.261</td>
</tr>
<tr>
<td>$tM/FFM$</td>
<td>0.382</td>
</tr>
<tr>
<td>$k_{(0,1)}$</td>
<td>-0.303</td>
</tr>
<tr>
<td>Flux$_{(2,1)}$</td>
<td>0.333</td>
</tr>
<tr>
<td>Plasma zinc flux</td>
<td>0.333</td>
</tr>
<tr>
<td>EZP$^2$</td>
<td>0.344</td>
</tr>
<tr>
<td>EZP/FFM$^2$</td>
<td>0.404</td>
</tr>
</tbody>
</table>

$^1 n = 47$ unless otherwise stated. FFM, fat-free mass; $k$, rate constant; $tM$, sum of $M1$, $M2$, and $M3$. Statistical analysis was performed by using simple linear regression and Spearman’s correlation coefficients; $P < 0.05$ indicates significance.

$^2 n = 48$.

**TABLE 4**

Effect of 6 mo of zinc supplementation on exchangeable zinc pools (EZPs) in men in the placebo and zinc-supplemented groups according to the Miller model

<table>
<thead>
<tr>
<th></th>
<th>Placebo group (n = 16)</th>
<th>15-mg Zn group (n = 16)</th>
<th>30-mg Zn group (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EZP (mg)</td>
<td>143 ± 15$^a$</td>
<td>154 ± 11$^b$</td>
<td>158 ± 21$^b$</td>
</tr>
<tr>
<td>EZP/FFM</td>
<td>2.53 ± 0.24$^a$</td>
<td>2.73 ± 0.22$^b$</td>
<td>2.86 ± 0.41$^b$</td>
</tr>
<tr>
<td>$t_{1/2}$ (b)</td>
<td>301 ± 50</td>
<td>326 ± 44</td>
<td>353 ± 102</td>
</tr>
<tr>
<td>Turnover (b)</td>
<td>434 ± 72</td>
<td>470 ± 64</td>
<td>510 ± 148</td>
</tr>
<tr>
<td>Flow rate out (mg/h)</td>
<td>0.337 ± 0.052</td>
<td>0.332 ± 0.043</td>
<td>0.323 ± 0.058</td>
</tr>
</tbody>
</table>

$^a n = 16$, FFM, fat-free mass; $t_{1/2}$, half-life. Means in a row with different superscript letters are significantly different, $P < 0.05$ (one-factor ANOVA followed by a Tukey-Kramer multiple comparisons test or a Kruskal-Wallis test).
on exchangeable zinc pool masses in late-middle-aged men. Kinetic analyses of zinc metabolism were performed over the past 2 decades with the use of radioactive $^{65}$Zn as a tracer (13, 19, 20). These studies have provided new insight into the mechanisms of zinc metabolism regulation and zinc homeostasis. Methods that incorporated the use of a stable isotope were validated with improved analytic techniques and produced zinc kinetic data similar to the data obtained with $^{65}$Zn (21). Large and elaborate compartmental models have been proposed to explore the underlying physiology of zinc (15, 16, 21, 22), but these models require numerous blood, urinary, and fecal samples and complex mathematical modeling. In our study, a simple 3-compartmental model, as proposed by Pinna et al (7), was applied instead of the more complex multicompartmental models mentioned above. We tested whether a simple model would be sufficient to determine the effect of zinc supplementation on zinc status. The model used in our study was found to be adequate because the plasma concentration of the zinc isotope fitted well to the sum of 3 exponentials. If it is difficult to identify a physiologic location for each zinc pool used in the present study, but it has been speculated from animal (23) and human (13, 20) studies that pool 1 represents plasma zinc, whereas pools 2 and 3 represent zinc in erythrocytes, liver, intestine, and bone. In our study, the mass of pool 1 was fixed to achieve the best possible computer fit of the data by calculating the mass on the basis of plasma zinc concentration and plasma volume. This was possible because Yokoi et al (24) showed that the mass of pool 1 is highly correlated with plasma zinc concentrations ($P = 0.0099$). Wastney et al (13) estimated that total body zinc is $\approx 1.6$ g, 6% of which exchanges rapidly with the plasma and the remaining 94% of which is located largely in muscle and bone. Our results agree with this hypothesis because the exchangeable zinc pool masses were <10% of the total body zinc in our late-middle-aged subjects.

The characteristics of the combined EZP masses, which exchange with plasma zinc within 48 h of tracer administration (11), were also examined. This approach, the use of plasma tracer enrichment data between day 3 and day 7, is simpler than the frequent early sampling that is required for the compartmental analysis. This method, however, is accurate only if the following 2 conditions are met: 1) the loss of tracer from EZP occurs at a monoeXponential rate from the moment of tracer administration to the end of the measurement period and 2) the tracer is homogeneously mixed throughout the EZP during the measurement period (20). This method thus overestimates the EZP mass because the initial rapid loss of the tracer from plasma is not accounted for by extrapolation of the monoeXponential loss rate during the measurement period and because it is extremely unlikely that the tracer exists at equal concentrations in all compartments of the EZP at a given time (11). In fact, when estimates from both approaches used in the present study were compared, the EZP approach overestimated the EZP mass by $\approx 15$–20% compared with the mass estimated by the multicompartmental model. In a previous study, in which we determined exchangeable magnesium pool masses by compartmental analysis and by the Miller approach (11), we observed that the exchangeable magnesium pool was overestimated by $\approx 45$–50% (18). The higher overestimation of magnesium was undoubtedly due to differences in the homeostasis mechanisms of zinc and magnesium in humans.

We observed that the exchangeable zinc pools were 33% lower in our late-middle-aged population than in healthy adults (7). In our study, the masses of these pools were negatively correlated with age in the placebo group. Because FFM is lower in elderly subjects and because zinc is an integral part of the protein mass in lean tissue, the lower exchangeable zinc pool masses observed in our late-middle-aged subjects may have been the result of a lower FFM. However, when expressed as FFM, the exchangeable zinc pool masses determined with the kinetic method were still negatively correlated with age. Moreover, no correlation was observed between FFM and exchangeable zinc pool mass. The low dietary zinc intake in the nonsupplemented subjects may also explain the lower exchangeable zinc pool mass. Indeed, although the dietary zinc intake of our late-middle-aged subjects was only $\approx 6$ mg/d, their plasma zinc concentrations were normal [>10.7 µmol/L (25)]. The recommended daily intake of zinc in France is 8 mg/d for elderly people who eat a diet rich in animal products (26), whereas in the United States the recommended intake is 11 mg/d for the same population (27).

Zinc supplementation for 6 mo significantly increased exchangeable zinc pool masses, irrespective of the approach used to estimate the pool masses. Very few studies have examined the effect of moderate dietary zinc intake on zinc tracer kinetics, and in some cases the zinc supplementation was very high [100 mg Zn/d for $\approx 9$ mo (13, 19) compared with 15 or 30 mg Zn/d for 6 mo in our study]. In these complex kinetic models, rapidly exchangeable zinc pool masses increased by 26% (19) or up to 85% (13) when oral zinc intake was increased 11-fold. Moreover, the plasma zinc mass increased by 37% (19). In our study, we showed that the exchangeable zinc pool mass increased by $\approx 10$–15% with a moderate zinc supplementation of 30 mg/d. Several studies have explored the effects of zinc depletion alone and the effects of zinc depletion and repletion in humans (6–11). Although low exchangeable zinc pool masses were observed with severe zinc depletion, no modification was observed with marginal zinc depletion. Moreover, zinc pool masses were not totally restored after 1 mo of zinc repletion. Therefore, it is possible that the exchangeable zinc pool mass changes only when there is a critical loss of whole-body zinc or after long-term zinc supplementation when a new steady state is reached. A human study indicated that zinc losses and zinc absorption may be adjusted to match zinc intake over a 10-fold range (28). The major sites of whole-body zinc homeostasis are in the gastrointestinal tract, and homeostatic mechanisms involve both zinc absorption and excretion of endogenous zinc in the feces (29). However, mechanisms that regulate total absorbed zinc are limited and may result in exchangeable zinc pool masses that vary directly with the quantity of zinc ingested and absorbed (30). In our study, exchangeable zinc pool masses correlated strongly with zinc intake, as previously observed in a zinc-depletion study (9), in a zinc depletion and repletion study (11), and in a study in breastfed infants (31).

No significant changes in the plasma distribution rate constants were detected, as previously observed with acute zinc depletion (6), which suggests that the determination of zinc pool kinetics is not an effective means of establishing zinc status. In addition, the half-lives of the zinc pools did not change significantly with zinc supplementation. Plasma zinc flux was significantly increased with zinc supplementation and correlated strongly with zinc intake, and previous studies have reported that plasma zinc flux declines with poor zinc status (6, 7). This kinetic
parameter appears to be an interesting marker of zinc status, as suggested by Yokoi et al (32). Because plasma turnover was not modified with zinc supplementation, the increased plasma zinc flux can be explained by the increase in plasma zinc concentrations after zinc supplementation. As observed in more complex kinetic studies (13, 15), the plasma zinc pool exchanged ≈ 140 times/day.

It is useful to consider whether kinetic markers provide a greater insight into zinc status than do biochemical indicators. As observed in acute zinc depletion (6), plasma zinc concentrations appear to be a better indicator of zinc status than do total exchangeable zinc pool masses. In fact, although plasma zinc concentrations increased up to 22% with zinc supplementation in the present study, zinc concentrations of the total exchangeable zinc pool mass barely increased to 15% with the kinetic method and barely increased to 10% with the EZP method. However, a regression analysis showed that of all the variables measured (both kinetic and biochemical), $M1/FFM$ had the strongest relation to dietary intake, followed by $tM/FFM$, $tM$, $EZP/FFM$, and plasma zinc concentration. This finding is different from what was observed with zinc depletion, where plasma zinc concentrations followed by plasma zinc flux were the parameters with the best relations to dietary intake (5). Another important point is that there are many physiologic and pathological conditions that may increase or decrease plasma zinc concentrations independent of the intake or intestinal absorption of zinc, whereas exchangeable zinc pool masses may not be as affected under these conditions.

In conclusion, our data showed that long-term supplementation with 2 moderate doses of zinc is an efficient way of increasing exchangeable zinc pool masses in late-middle-aged men. The changes in exchangeable zinc pool masses were smaller than the changes in plasma zinc concentration; thus, exchangeable zinc pool masses may not be as sensitive as are plasma zinc concentrations for the assessment of zinc status. Moreover, these exchangeable zinc pool masses decreased with age and correlated with total dietary zinc intake. This may mean that the actual recommended intake of zinc in humans is insufficient in France and should be increased to agree with the recommended intake in the United States.

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CF-C participated in the data analysis and the writing of the manuscript. NM participated in the subject selection, experiment design, and data collection. MR and JCF participated in the data collection and analysis. MB-B participated in the dietary zinc estimation. MA, AM, and KDC provided significant advice. CC participated in the experimental design and the writing of the manuscript. None of the authors had any financial or personal conflicts of interest.

REFERENCES


Choline supplemented as phosphatidylcholine decreases fasting and postmethionine-loading plasma homocysteine concentrations in healthy men¹–³

Margreet R Olthof, Elizabeth J Brink, Martijn B Katan, and Petra Verhoef

ABSTRACT

Background: A high homocysteine concentration is a potential risk factor for cardiovascular disease that can be reduced through betaine supplementation. Choline is the precursor for betaine, but the effects of choline supplementation on plasma total homocysteine (tHcy) concentrations in healthy humans are unknown.

Objective: The objective was to investigate whether supplementation with phosphatidylcholine, the form in which choline occurs in foods, reduces fasting and postmethionine-loading concentrations of plasma tHcy in healthy men with mildly elevated plasma tHcy concentrations.

Design: In a crossover study, 26 men ingested ≈2.6 g choline/d (as phosphatidylcholine) or a placebo oil mixture for 2 wk in random order. Fatty acid composition and fat content were similar for both treatments. A methionine-loading test was performed on the first and last days of each supplementation period.

Results: Phosphatidylcholine supplementation for 2 wk decreased mean fasting plasma tHcy by 18% (−3.0 μmol/L; 95% CI: −3.9, −2.1 μmol/L). On the first day of supplementation, a single dose of phosphatidylcholine containing 1.5 g choline reduced the postmethionine-loading increase in tHcy by 15% (−4.8 μmol/L; 95% CI: −6.8, −2.8 μmol/L). Phosphatidylcholine supplementation for 2 wk reduced the postmethionine-loading increase in tHcy by 29% (−9.2 μmol/L; 95% CI: −11.3, −7.2 μmol/L). All changes were relative to placebo.


KEY WORDS Choline, phosphatidylcholine, methionine loading, homocysteine, humans

INTRODUCTION

Choline is an important nutrient throughout life. It is the precursor for the neurotransmitter acetylcholine and for phosphatidylcholine, a structural component of VLDL, which is essential for normal lipid-cholesterol transport. Furthermore, choline is a source of labile methyl groups (1). For a long time, choline was considered a dispensable nutrient because it can be endogenously synthesized through sequential methylation of phosphatidylethanolamine to form phosphatidylcholine, with S-adenosylmethionine as the methyl donor (2, 3). However, studies have shown that humans who ingest a choline-deficient diet develop liver and kidney problems (4–6). Thus, choline is an important dietary nutrient, and an adequate intake of choline is defined as 425 mg/d for women and 550 mg/d for men (7).

Choline is present in the human diet primarily as lecithin, which is the common name for phosphatidylcholine. Intake of the choline moiety from foods is estimated at 0.3–1 g/d, and the main food sources are eggs, liver, soybeans, and pork (3, 8, 9). Choline becomes a source of labile methyl groups when it is converted into betaine (Figure 1). This conversion occurs mainly in the liver and kidney and is irreversible (1, 10). Betaine donates its methyl group to homocysteine to form methionine in a reaction catalyzed by the enzyme betaine-homocysteine methyltransferase. A high plasma homocysteine concentration is associated with a greater risk of cardiovascular disease (CVD), but whether this relation is causal is still uncertain (11). Supplementation with betaine lowers plasma homocysteine concentrations in hyperhomocysteinemic subjects (12, 13) and in subjects with normal homocysteine concentrations (14–16). Choline has been used in the past as a homocysteine-lowering therapy for hyperhomocysteinemic patients with genetic defects in their homocysteine metabolism who had not responded to treatment with vitamin B-6 or folic acid (17). Dudman et al (18) found that choline or betaine treatment normalized concentrations of homocysteine after methionine loading in some but not all patients with CVD and impaired homocysteine metabolism. The effects

¹ From the Wageningen Centre for Food Sciences and the Division of Human Nutrition, Wageningen University, Wageningen, Netherlands (MRO, MBK, and PV), and the Department of Physiological Sciences, Netherlands Organisation for Applied Scientific Research (TNO) Quality of Life, Zeist, Netherlands (EJB).
² Supported by the Wageningen Centre for Food Sciences, an alliance of Dutch food industry and research institutes [the University of Maastricht, the Netherlands Organisation for Applied Scientific Research (TNO) Quality of Life, and Wageningen University and Research Centre] that receives funding from the Dutch government. Unilever Research Laboratory donated the placebo oil used in the study.
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of choline supplementation on plasma homocysteine concentrations in healthy subjects are unknown. Therefore, we investigated the effect of supplementation with choline (as phosphatidylcholine) on fasting and postmethionine-loading plasma concentrations of homocysteine in healthy men.

SUBJECTS AND METHODS

Subjects

Subjects were recruited from the pool of volunteers registered at the Netherlands Organisation for Applied Scientific Research (TNO) Quality of Life (Zeist, Netherlands) and by advertisements in local newspapers. Eligible volunteers were healthy as assessed by physical examination, a general health and lifestyle questionnaire, blood pressure measurement, routine clinical laboratory tests, and blood analyses of tHcy and B vitamins. Plasma tHcy concentrations were <26 μmol/L. Volunteers had no history of CVD and had not used vitamin B supplements, lecithin, or supplements containing choline, choline derivatives, or betaine > 1 time/wk during the month preceding screening.

Of 48 eligible men, 26 men aged 50–71 y with the highest plasma tHcy concentrations (range: 11.0–23.1 μmol/L) were included in this study. Subject characteristics are shown in Table 1. All subjects completed the study. Written informed consent was obtained from all subjects. The study was conducted according to Good Clinical Practice guidelines at TNO Quality of Life. The local medical ethics committee approved the protocol.

Study design

In this double-blind, placebo-controlled, crossover study, the subjects were randomly assigned to 1 of 2 treatment orders. Randomization was stratified by plasma tHcy concentrations at screening and by smoking habits. Thirteen subjects began phosphatidylcholine treatment, and the other 13 began placebo treatment, both for 2 wk. After a 2-wk washout period, the treatments were reversed. Treatments consisted of ingestion of 34.0 g of a

![Figure 1. The role of choline in homocysteine metabolism. Box: structure of phosphatidylcholine](image)

TABLE 1

<table>
<thead>
<tr>
<th>Subject characteristics at screening</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>61 ± 6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.7 ± 2.2</td>
</tr>
<tr>
<td>tHcy (μmol/L)</td>
<td>14.7 ± 3.4</td>
</tr>
<tr>
<td>Vitamin B-6 (nmol/L)</td>
<td>64 ± 44</td>
</tr>
<tr>
<td>Vitamin B-12 (pmol/L)</td>
<td>236 ± 61</td>
</tr>
<tr>
<td>Folate (nmol/L)</td>
<td>11.7 ± 3.9</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>73 ± 16</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>21 ± 8</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>21 ± 6</td>
</tr>
<tr>
<td>γ-GT (U/L)</td>
<td>25.7 ± 11.0</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>88 ± 11</td>
</tr>
</tbody>
</table>

1 All values are \( \bar{x} \pm SD; n = 26. All measurements in blood were taken after an overnight fast. tHcy, total homocysteine; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GT, glutamyltransferase.
The phosphatidylcholine supplement as supplied by Nutrasal LLC (Oxford, CT) consisted of 80% soybean lecithin extract (Phosal 75A), 18% medium-chain triacylglycerol mix, 1.5% anethole, and traces of D-α-tocopherol and paraben blend. The soybean lecithin extract consisted of 8% moisture, 3% ash, 10% protein, 71% fat (including phosphatidylcholine), 9% carbohydrates, and traces of vitamins and minerals. Folic acid content of the extract was 195 μg/kg, which is ~5 μg/34 g phosphatidylcholine oil (calculated from data provided by the manufacturer). This amount is negligible relative to the total daily intake of folate (100–200 μg/d).

2.5 g choline in 34 g of the phosphatidylcholine supplement (corresponding to 1.5 g choline) or an equivalent amount of placebo.

The 20 g of phosphatidylcholine used was the largest amount that delivered a dose of 2.6 g choline (day 15) of each treatment period. On these days, a fasting blood sample was collected from each subject, and then subjects ingested 100 mg L-methionine/kg body wt (Methioninum apyrogen; BUFA BV Pharmaceutical Products, Uitgeest, Netherlands). Methionine was dissolved in a glass of orange juice and ingested together with a standardized breakfast low in methionine and with 20 g of the phosphatidylcholine supplement (corresponding to 1.5 g choline) or an equivalent amount of placebo.

The amount of phosChol was measured in 2 different laboratories. Koc et al (19) measured choline as phosphatidylcholine in 2 samples. Phosphatidylcholine concentrations in the 2 samples were 800 and 801 μmol/g sample; this represents ~20.6 g phosphatidylcholine (molecular weight 758) or ~2.8 g choline in 34 g PhosChol. Total choline content was also measured in 4 samples of PhosChol at TNO (see Subjects and Methods). Mean choline content was 74.8 mg/g (range: 71–77 mg/g); this represents 2.5 g choline in 34 g PhosChol. The mean choline content from all 6 samples analyzed was 2.6 g/d.

Fatty acids were measured by gas chromatography of fatty acid methyl esters (20). Trimonadecanoin (19:0) was used as a reference compound to calculate the amounts of individual fatty acids.

Blood collection

Venous blood was taken from the antecubital vein after an overnight fast on days 1, 13, and 15 of each treatment period. In addition, blood samples were obtained 6 h after methionine loading on days 1 and 15 of each treatment period. Blood for analysis of tHcy and vitamin B-6 was collected in evacuated tubes containing EDTA. Samples were mixed and put on ice immediately after collection. Within 30 min, samples were centrifuged for 15 min at 2000 × g at 4 °C. For analyses of vitamins B-12 and folic acid; blood lipids; and alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, γ-glutamyltransferase, and creatinine, blood was collected in evacuated tubes containing clot activator and a gel to separate serum and cells. Approximately 30 min after collection, samples were centrifuged for 15 min at 2000 × g at 4 °C. All samples were stored at −70 °C. Samples were coded to hide the identity and treatment of subjects. All samples obtained from one subject were analyzed in the same run.
Biochemical analyses

Plasma tHcy concentrations were measured in fasting blood samples that were collected on days 1, 13, and 15 of each treatment period and in nonfasting blood samples collected after the methionine loads on days 1 and 15 of each treatment period. The concentrations of tHcy (sum of all oxidized and reduced forms of homocysteine) were measured by using HPLC with fluorescence detection (21). Within- and between-run CVs were 3.5% and 5%, and 95% CIs were calculated. Statistical analyses were carried out using SAS software (version 8.1; SAS Institute Inc, Cary, NC).

RESULTS

Fasting and postmethionine-loading total homocysteine

Fasting plasma tHcy was 18% lower after subjects had ingested phosphatidylcholine for 2 wk than after placebo treatment (Table 3). Treatment effects were not influenced by treatment order ($P = 0.59$).

On the first day of phosphatidylcholine supplementation (ie, after a single dose), the increase in plasma tHcy 6 h after methionine loading was 15% lower than that on the first day of placebo treatment (Table 3). On the last day of phosphatidylcholine supplementation, the increase in plasma tHcy 6 h after methionine loading was 29% lower than that after placebo treatment (Table 3). In addition, the effect of a dose of phosphatidylcholine with a methionine load on postmethionine-loading homocysteine concentrations on the first day was smaller than the effect on the

| TABLE 3 | Fasting plasma total homocysteine (tHcy) and the increase in plasma tHcy after methionine loading in 26 healthy men after their ingestion of placebo and of phosphatidylcholine (corresponding to 2.6 g choline/d) in a crossover design |
|-----------------|-----------------|-----------------|
| **Placebo** | **Phosphatidylcholine** | **Difference from placebo** |
| Fasting (µmol/L)$^1$ | | |
| First day of supplementation | 16.5 ± 4.2$^2$ | 15.6 ± 4.0 | −0.9 (−1.8, −0.0)$^3$,4 |
| After 2 wk of supplementation | 16.6 ± 4.0 | 13.6 ± 2.5$^5$ | −3.0 (−3.9, −2.1)$^6$ |
| 6-h Postmethionine load (µmol/L)$^7$,8 | | | |
| First day of supplementation | 31.8 ± 7.0 | 27.0 ± 6.1 | −4.8 (−6.8, −2.8)$^6$ |
| After 2 wk of supplementation$^9$ | 31.6 ± 6.0 | 22.3 ± 3.3$^{10}$ | −9.2 (−11.3, −7.2)$^6$ |

$^1$ Treatment × time interaction, $P = 0.001$ (split-plot ANOVA): thus, the effect on the first day of supplementation was significantly different from the effect after 2 wk.

$^2$ $\bar{x} ± SD$ (all such values).

$^3$ $\bar{r}$; 95% CI in parentheses (all such values).

$^4$ $P = 0.04$ (split-plot ANOVA).

$^5$ Significantly different from fasting tHcy on day 1 of phosphatidylcholine treatment, $P < 0.001$ (split-plot ANOVA).

$^6$ $P < 0.0001$ (split-plot ANOVA).

$^7$ The increase in plasma tHcy 6 h after methionine loading is the difference between the value obtained immediately before methionine loading and that obtained 6 h after methionine loading.

$^8$ Treatment × time interaction, $P = 0.003$ (split-plot ANOVA): thus, the effect on the first day of supplementation was significantly different from the effect after 2 wk.

$^9$ $n = 25$ because 1 sample was lost during processing.

$^{10}$ Significantly different from postmethionine-loading tHcy on day 1 of phosphatidylcholine treatment, $P < 0.0001$ (split-plot ANOVA).
last day of supplementation (Table 3). Treatment effects were not influenced by treatment order (P = 0.35).

B vitamins

Serum folate concentrations were significantly lower after phosphatidylcholine (10.9 ± 3.4 nmol/L) than after placebo (11.8 ± 3.1 nmol/L) treatment (P = 0.04). Concentrations of vitamin B-6 were significantly higher after phosphatidylcholine (53 ± 23 nmol/L) than after placebo (49 ± 17 nmol/L) treatment (P = 0.05). Vitamin B-12 concentrations did not differ significantly between the 2 treatment periods.

Liver and kidney function indicators, serum lipids, and body weight

Serum alkaline phosphatase concentrations were significantly lower after phosphatidylcholine (64 ± 13 U/L) than after placebo (68 ± 14 U/L) treatment (P = 0.001). Concentrations of alanine aminotransferase, aspartate aminotransferase, γ-glutamyltransferase, and creatinine did not differ significantly between treatments (data not shown). Serum triacylglycerol concentrations were significantly higher after subjects ingested phosphatidylcholine for 2 wk (2.00 ± 1.27 mmol/L) than after they ingested placebo (1.77 ± 1.15 mmol/L) (statistical analyses performed on log-transformed values, P = 0.001). Serum concentrations of total, LDL, and HDL cholesterol did not differ significantly between treatment periods.

Body weight increased by 0.4 kg during each of the 2-wk treatment periods (P = 0.007), and the increase did not differ significantly between treatments (P = 0.95). During the 2-wk washout period, subjects lost the weight that they had gained during the preceding 2-wk treatment, so that, at the start of the second supplementation period, they were back to their starting weights (data not shown).

DISCUSSION

Effects on total homocysteine

We showed that supplementation with phosphatidylcholine lowers fasting and postmethionine-loading plasma tHcy concentrations in healthy men with mildly elevated plasma tHcy concentrations. A single dose of phosphatidylcholine together with a methionine load acutely reduced the increase in tHcy after methionine loading, which indicates that production of betaine from supplemental choline is quick (10, 26, 27). Because we matched the fatty acid content and composition of the supplements, we may assume that the effects we found are solely due to the choline moiety of phosphatidylcholine. Both fasting and postmethionine-loading tHcy concentrations are predictors of CVD risk (28–30). If homocysteine truly is causally involved, supplementation with choline from phosphatidylcholine might be a novel dietary way to decrease CVD risk (11, 31).

The decrease in plasma tHcy through choline is most likely mediated through increased betaine-dependent remethylation of homocysteine into methionine. Excess choline is irreversibly converted into betaine by the enzyme choline oxidase (10, 32, 33), which increases betaine-dependent remethylation and leads to homocysteine lowering (Figure 1). An alternative mechanism could involve a reduction in endogenous production of phosphatidylcholine via the phosphatidylethanolamine N-methyltransferase pathway when phosphatidylcholine is supplemented, which in turn may lead to lower homocysteine concentrations (34). Approximately 30% of the phosphatidylcholine is formed through sequential methylation of phosphatidylethanolamine, which generates 3 homocysteine molecules for each phosphatidylcholine molecule synthesized (35). However, whether this mechanism can explain the homocysteine-lowering effects of phosphatidylcholine is not known, and that possibility should be investigated.

The effects of choline, betaine, and folic acid on plasma total homocysteine

We compared the effects of phosphatidylcholine, betaine, and folic acid supplementation on plasma tHcy concentrations by using data from studies previously done by our group in the same setting and laboratory (14, 15, 36). Supplementation with phosphatidylcholine, corresponding to 2.6 g choline/d, reduced fasting tHcy by 18% after 2 wk, which is similar to the reduction seen after 2 wk treatment with 1.5–3 g betaine/d (14). A single dose of phosphatidylcholine, corresponding to ≈1.5 g choline/d, reduced the postmethionine-loading increase in tHcy as much as did a single 0.75-g dose of betaine (14). The acute effects on postmethionine-loading plasma tHcy thus appear somewhat more efficient with betaine than with phosphatidylcholine supplementation. This seems plausible because, once absorbed, betaine is directly available as a methyl donor, whereas choline first has to be oxidized to betaine. Lowering of fasting plasma tHcy after phosphatidylcholine supplementation was similar to that after supplementation with ≈400 µg folic acid/d (36). Folic acid does not affect postmethionine-loading plasma tHcy, but phosphatidylcholine and betaine do (14, 15, 37). Thus, our results imply that betaine and phosphatidylcholine, given as supplements, can serve as alternatives to folic acid as a homocysteine-lowering agent. In addition, phosphatidylcholine and betaine supplementation might temper tHcy increases after a meal, whereas folic acid does not.

Effect of phosphatidylcholine on B vitamins and on kidney and liver function indicators

Folic acid–dependent remethylation and choline- or betaine-dependent remethylation are interrelated (38). In humans and animals, a choline-deficient diet led to low folate concentrations, which were restored with the administration of choline (39–42). Conversely, when the diet is deficient in folate, choline concentrations are low, but choline status is restored with folate repletion (39, 43–45). Contrary to our expectations, we found that phosphatidylcholine supplementation decreased serum folate concentrations and increased vitamin B-6 concentrations in blood. However, the effects were small, and this probably is a chance finding.

Choline deficiency leads to liver problems both in healthy humans and in humans on total parenteral nutrition that is choline poor (4–6). We found that phosphatidylcholine supplementation did not greatly affect liver or kidney function. However, the small increase in triacylglycerol concentrations on phosphatidylcholine supplementation should be investigated further.

Study limitations

We tested the effects of phosphatidylcholine supplementation on plasma homocysteine only in men. From animal studies, it appears that females are more resistant to induced choline deficiency than are males, probably because of females’ enhanced
capacity to form phosphatidylcholine endogenously (46–48). It is not known whether supplementation with phosphatidylcholine will affect plasma homocysteine concentrations differently in men and in women who are choline replete.

We supplied choline in the form of phosphatidylcholine and not as free choline, mainly because phosphatidylcholine is the form in which choline occurs in foods. In addition, ingestion of phosphatidylcholine leads to a prolonged and greater increase in serum choline concentrations than does ingestion of free choline (as choline chloride) (49), and therefore the former might be more effective in lowering tHcy. Furthermore, unlike the ingestion of high doses of free choline, that of phosphatidylcholine does not lead to the undesirable formation of trimethylamines that makes persons who ingest large amounts of choline smell of fish (50). Nevertheless, for the production of foods with extra choline or for supplementation purposes, choline salts will be more feasible than will phosphatidylcholine. In addition, phosphatidylcholine provides extra energy intake due to the fatty acid component, which can be avoided when free choline is ingested.

Furthermore, we supplied a high dose of choline relative to dietary intake of choline (estimated at 0.3–1 g/d). The homocysteine-lowering potential of choline doses in the range of dietary intake of choline (estimated at 0.3–1 g/d). The energy content of the supplements (dietary intake is as yet unknown. Because of the substantial homocysteine-lowering potential of choline doses in the range of dietary intake of choline (estimated at 0.3–1 g/d) and therefore the former might be more effective in lowering tHcy. Furthermore, unlike the ingestion of high doses of free choline, that of phosphatidylcholine does not lead to the undesirable formation of trimethylamines that makes persons who ingest large amounts of choline smell of fish (50). Nevertheless, for the production of foods with extra choline or for supplementation purposes, choline salts will be more feasible than will phosphatidylcholine. In addition, phosphatidylcholine provides extra energy intake due to the fatty acid component, which can be avoided when free choline is ingested.

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Furthermore, we supplied a high dose of choline relative to dietary intake of choline (estimated at 0.3–1 g/d). The homocysteine-lowering potential of choline doses in the range of dietary intake is as yet unknown. Because of the substantial energy content of the supplements (~230 kcal/d), the body weight of the subjects increased by ~0.4 kg during both intervention periods. Apparently, the subjects did not (completely) compensate for the extra energy intake from the supplements. However, weight changes did not affect our results because the increases in weight were similar during the 2 intervention periods.

Conclusion

We conclude that phosphatidylcholine supplementation is as effective as betaine and folic acid in lowering fasting tHcy. We expect that betaine or choline supplementation in combination with folic acid supplementation or fortification will augment the tHcy-lowering effect of folic acid, but that possibility remains to be investigated. Both phosphatidylcholine and betaine supplements lower postmethionine-loading plasma tHcy concentrations, but folic acid does not. If homocysteine is causally related to CVD, a diet rich in (phosphatidyl)choline or supplements containing (phosphatidyl)choline might prove to be beneficial. However, the effects of phosphatidylcholine on other risk factors for CVD, including serum triacylglycerols, should first be studied in greater detail.

We thank the volunteers for their participation, all those involved at TNO Quality of Life for their dedication, and the laboratory staff at the Division of Human Nutrition, Wageningen University, for laboratory analyses. All authors participated in the design of the study, interpretation of the data, and writing of the manuscript. EJB also participated in the conduct of the study. None of the authors had any personal or financial conflicts of interest.

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Zinc absorption and kinetics during pregnancy and lactation in Brazilian women¹–⁴

Carmen M Donangelo, Carmiña L Vargas Zapata, Leslie R Woodhouse, David M Shames, Ratna Mukherjea, and Janet C King

ABSTRACT

Background: Adjustments in zinc absorption and endogenous excretion maintain zinc homeostasis in nonpregnant adults fed low-zinc diets. The effects on zinc homeostasis of a low zinc intake during pregnancy and lactation have not been described in a longitudinal study.

Objective: We examined longitudinal changes in fractional zinc absorption (FZA) and zinc kinetics in 10 healthy Brazilian women who habitually consumed a marginal zinc diet (≈9 mg Zn/d).

Design: Zinc status was measured at 10–12 (early pregnancy; EP) and 34–36 (late pregnancy; LP) wk of pregnancy and at 7–8 wk after delivery (early lactation; EL). Zinc kinetics and FZA were studied by using stable isotopic tracers.

Results: Zinc intake averaged 9 ± 3 mg/d throughout the study. FZA increased from 29 ± 6% at EP to 43 ± 10% at LP and to 39 ± 13% at EL (P < 0.05). FZA was inversely related to plasma zinc at EL (r = −0.73, P = 0.02) and LP (r = −0.72, P = 0.07). Plasma zinc mass was 23% greater at LP than at EP or EL (P < 0.05). The amount of zinc (mg/d) that fluxed between plasma and the most-rapidly-turning-over extravascular pool was 53% greater at LP than at EP or EL (P < 0.05). The zinc flux between plasma and the less-rapidly-turning-over zinc pool at EL was 27% greater than that at EP or LP, but this difference was not significant.

Conclusions: FZA increased significantly in women with marginal zinc intakes during pregnancy and lactation; the increase was higher in women with low plasma zinc. Plasma zinc was distributed into a different exchangeable pool at LP than at EL. Am J Clin Nutr 2005;82:118–24.

KEY WORDS Pregnancy, lactation, zinc absorption, zinc kinetics, stable isotopes, Brazilian women

INTRODUCTION

The need for zinc increases during pregnancy and lactation because of the greater demands of normal embryogenesis, fetal growth, and milk secretion. The total demand in a full-term pregnancy is ≈100 mg Zn; the need parallels fetal growth and reaches a peak increase of 1 mg absorbed Zn/d in the third trimester (1). During lactation, the additional demand for absorbed zinc is highest in the first 2 mo of lactation, when milk zinc concentrations are high (2). These increased zinc needs could be met by an increase in zinc intake or by adjustments in zinc homeostasis. Because women do not typically increase their zinc intake during pregnancy and lactation (3), adjustments in zinc absorption, excretion, tissue distribution, or all 3 must occur to meet these greater demands for zinc.

The efficiency of zinc absorption has been measured in pregnant (4) and lactating (5–7) women with marginal zinc intakes (<10 mg/d) and in women consuming ≈15 mg Zn/d during pregnancy and lactation (8). These studies showed that the efficiency of zinc absorption increased during lactation, especially among women with marginal zinc intakes. However, the effect of pregnancy on the efficiency of zinc absorption was inconclusive.

Additional homeostatic adjustments, such as reduced urinary zinc excretion, reduced endogenous fecal zinc losses, and an increased mobilization of zinc from bone and other tissues, could theoretically contribute to the greater need for zinc during pregnancy and lactation in women with low zinc intakes (3, 9, 10). However, studies of the effects of pregnancy and lactation on these mechanisms are limited. A study in China showed that intestinal conservation of endogenous zinc was a major factor in achieving zinc homeostasis in lactating women who consumed only 7.6 mg Zn/d (7). A preliminary zinc kinetic study of lactating women of the Amazon River Valley (Brazil) who consumed diets providing 8.4 mg Zn/d suggested that the lactation state increased the plasma zinc turnover rate and decreased the size of the exchangeable zinc pool in these women (6). The development of detailed compartmental models of zinc metabolism in nonpregnant, nonlactating women by using oral and intravenous stable isotopes provided a means of conducting more detailed studies of zinc homeostasis during pregnancy and lactation (11). Therefore, the purpose of the current study was to ascertain

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changes in fractional zinc absorption (FZA), urinary zinc excretion, and zinc kinetics by using a 3-compartment model during pregnancy and lactation in a group of healthy Brazilian women who habitually consumed a diet with marginal zinc content.

SUBJECTS AND METHODS

Subjects

Subjects were recruited for the study at their first prenatal visit at the Maternidade Escola of the Federal University of Rio de Janeiro, Brazil. Ten adult women of low socioeconomic status agreed to participate in the study. The women were healthy, nonsmoking, physically inactive multigravidae, and they had had no health problems or complications during pregnancy. All of the women were of mixed black and white race. Iron supplements (50–100 mg Fe/d) were given to all of the women during the second half of pregnancy (22–35 wk of gestation) as part of their routine prenatal care, but none of the women took zinc or other vitamin or mineral supplements. No iron supplements were used after delivery. The women were advised to consume their usual diets throughout the study.

Written informed consent was obtained from all subjects. The study was approved by the Committee for the Protection of Human Subjects at the University of California, Berkeley, and by the Ethical Committee of Maternidade Escola, Federal University of Rio de Janeiro.

Study design

Clinical studies of zinc homeostasis were done at 3 time points: week 10–12 (early pregnancy; EP) and week 34–36 (late pregnancy; LP) of pregnancy and 7–8 wk after delivery (early lactation; EL). Iron supplements were not taken during the clinical studies. All women were exclusively breastfeeding their infants at EL. Dietary zinc intake, fasting plasma and erythrocyte zinc concentrations, 24-h urinary zinc excretion, FZA, and zinc kinetics were measured at each time point.

Procedures and analysis

Dietary intake was assessed from weighed food-intake records kept by the subject on 3 consecutive days before the clinical study at each of the 3 time points. Subjects were carefully instructed and trained by one of the investigators (CVZ) in the food-weighing and -recording procedures. The dietary nutrient intake was estimated from the food records by using the Food Processor nutrient database (ESHA Research, Salem, OR) that was adapted for Brazilian foods with the use of published food-composition data (12). Phytate intake was estimated from published food phytate data (13).

Studies of FZA, plasma zinc kinetics, and urinary zinc excretion were performed after an overnight (10–12 h) fast at the Maternidade Escola of the Federal University of Rio de Janeiro by using a double isotopic tracer technique (8, 11). Highly enriched stable isotopic tracers, ie, $^{67}\text{Zn}_{\text{m}}$ (90.1% enriched with $^{67}\text{Zn}$) and $^{70}\text{Zn}_{\text{m}}$ (88.5% enriched with $^{70}\text{Zn}$) as zinc oxide (Oak Ridge National Laboratory, Oak Ridge, TN), were dissolved in concentrated hydrogen chloride (Optima; Fisher, Pittsburgh, PA; 3 μL HCl/mg ZnO). They were then diluted with triply deionized water to a final concentration of 1.0 mg $^{67}\text{Zn}_{\text{m}}$/mL or 0.4 mg $^{70}\text{Zn}_{\text{m}}$/mL and filtered through a 0.22-μm filter unit (Millipore Products Division, Bedford, MA). The solution for intravenous use was sterilized and pyrogen tested by the School of Pharmacy, University of California, San Francisco. Intravenous doses (1.0 mL) containing 0.4 mg $^{70}\text{Zn}_{\text{m}}$ were stored in individually sealed, sterile vials until they were used. The amounts of administered oral and intravenous tracer doses were calculated from the total zinc concentration of the solutions, which was measured by using atomic absorption flame spectrometry and the weight of the administered dose.

On the morning of the clinical study, the weight and height of the subjects were recorded, and an indwelling catheter was placed in the antecubital vein, from which a baseline (fasting) blood sample and all other blood samples were drawn with Monovette syringes (Sarstedt, Hayward, CA) that contained heparin-coated beads. The subjects then received a standard breakfast consisting of French bread (50 g), butter (10 g), and whole milk (50 mL) mixed with coffee (50 mL); this was followed by the ingestion of 1 mg $^{67}\text{Zn}_{\text{m}}$ in 50 mL water. The cup with the stable isotopic tracer solution was rinsed 3 times with water, and the water was consumed. The total zinc intake (breakfast plus oral zinc tracer) was 1.22 mg, and the total molar ratio of phytate to zinc was <1. Immediately after breakfast, a “butterfly” infusion set was used to infuse 0.4 mg $^{70}\text{Zn}_{\text{m}}$ (1.0 mL) over 1–2 min into the antecubital vein of the arm opposite that used for blood sampling. The butterfly tubing was flushed with 5 mL sterile saline solution to ensure that the entire tracer dose was infused. The exact amount of tracer solution infused was ascertained by weighing the syringe before and after the infusion. Blood samples (8 mL) were taken via the catheter at 4, 8, 12, 16, 20, 30, 45, and 60 min and 2, 3, 6, 9, 12, and 24 h after the $^{70}\text{Zn}_{\text{m}}$ infusion. A complete 24-h urine collection was obtained on the test day. Samples from the first urinary void were collected in the morning on days 3, 4, and 5 after the test.

Blood samples were refrigerated at 4 °C immediately after being drawn, and the plasma was separated within 2 h. Erythrocytes were obtained from the fasting baseline samples by removal of the buffy coat layer of packed red blood cells, washing of the cells twice with ice-cold 0.9% saline, and centrifugation at 800 × g for 10 min at 4 °C. The supernatant fluid was discarded, and an equal volume of ice-cold deionized water was added to the erythrocytes and mixed. Urine samples were weighed, and aliquots were acidified to a pH of 2.0 with trace-metal grade hydrogen chloride (Fisher). Aliquots of plasma, erythrocyte lysates, and urine were stored at −20 °C until they were analyzed for zinc. We used the cyanomethemoglobin method to measure hemoglobin; atomic absorption spectrometry to measure plasma, erythrocyte, and urinary zinc (8); a modified Lowry method to measure protein in erythrocytes (14); and the cadmium-hemoglobin affinity assay to measure metallothionein in erythrocytes (15).

Procedures used to prepare and analyze the urinary samples for mass spectrometric analysis by using inductively coupled plasma (ICP) were the same as those used previously in our laboratory (8). Briefly, urinary zinc was purified by ion exchange chromatography and submitted to ICP mass spectrometry (MS) (Sciex ELAN 5000 ICP-MS; Perkin Elmer, Norwalk, CT) for determination of the isotopic ratios of $^{67}\text{Zn}$ to $^{66}\text{Zn}$ and of $^{70}\text{Zn}$ to $^{66}\text{Zn}$. The isotopic ratios were then converted to tracer-tracee ratios (TTRs) as previously described (11), in which the TTR data highly enriched in $^{67}\text{Zn}$ and $^{70}\text{Zn}$ can be defined as $^{67}\text{Zn}_{\text{m}}\text{TTR}$ and $^{70}\text{Zn}_{\text{m}}\text{TTR}$, respectively.
FZA was measured by a novel modification of the double isotopic tracer ratio technique in urine as previously described (16, 17). Briefly, this technique is used to estimate FZA from spot urine samples obtained 3, 4, and 5 d after simultaneous oral and intravenous tracer administration. The 3 FZA determinations are then averaged for the reported result. Our current technique for FZA measurement, a slight modification of the above, takes advantage of the observation that both oral and intravenous TTR measurements ($^{67}\text{Zn}_{\text{TR}}$ and $^{70}\text{Zn}_{\text{TR}}$, respectively) disappear from the plasma by the same fractional loss rate, i.e., those measurements are describable by parallel lines on a semi-log plot when simple regression lines are fitted to the TTR data. The same parallel regression line pattern is also observed in sequential sampling of $^{67}\text{Zn}_{\text{TR}}$ and $^{70}\text{Zn}_{\text{TR}}$ data from spot urine samples. This technique is implemented by using the SAAM II computer program (version 1.2; SAAM Institute, Seattle, WA) in which individual exponential equations are fitted simultaneously to the spot urine $^{67}\text{Zn}_{\text{TR}}$ and $^{70}\text{Zn}_{\text{TR}}$ data, subject to the constraint that the exponential components are equal. Thus, the individual exponential equation for $^{67}\text{Zn}_{\text{TR}}(t)$ is

\[ ^{67}\text{Zn}_{\text{TR}}(t) = ^{67}\text{Int}_{\text{TR}} e^{-k_{67}t} \]  

and that for $^{70}\text{Zn}_{\text{TR}}(t)$ is

\[ ^{70}\text{Zn}_{\text{TR}}(t) = ^{70}\text{Int}_{\text{TR}} e^{-k_{70}t} \]

where $^{67}\text{Int}_{\text{TR}}$ and $^{70}\text{Int}_{\text{TR}}$ are the intercepts of the exponential equations describing the regression lines through the $^{67}\text{Zn}_{\text{TR}}$ and $^{70}\text{Zn}_{\text{TR}}$ data from spot urine over days 3–5, and where $k_{70} = k_{67}$.

FZA is then given by the equation

\[ \text{FZA} = \frac{^{67}\text{Int}_{\text{TR}}/\text{dose}^{67}\text{Zn}_{\text{u}}}{^{70}\text{Int}_{\text{TR}}/\text{dose}^{70}\text{Zn}_{\text{u}}} = \frac{^{67}\text{Int}_{\text{TR}}}{^{70}\text{Int}_{\text{TR}}} \times \frac{\text{dose}^{70}\text{Zn}_{\text{u}}}{\text{dose}^{67}\text{Zn}_{\text{u}}} \]  

Plasma samples were prepared for stable isotope analysis as done previously (11). TTRs in the plasma were calculated from isotopic ratios of $^{67}\text{Zn}$ to $^{66}\text{Zn}$ and of $^{70}\text{Zn}$ to $^{66}\text{Zn}$ that were measured by using ICP-MS. Plasma zinc concentrations, measured by ICP at each sampling time over 24 h, were corrected for the tracer mass $^{67}\text{Zn}_{\text{u}}$ and $^{70}\text{Zn}_{\text{u}}$ as described previously (11) and averaged for the best estimate of plasma zinc concentration. A 3-compartment model was fitted to the plasma $^{70}\text{Zn}_{\text{u}}$ data over 24 h by using the SAAM II modeling software (Figure 1). The 3 kinetically distinct zinc compartments of the model were $Q_1$, which represented the plasma zinc pool; $Q_2$, which represented a rapidly-turning-over tissue zinc pool; and $Q_3$, which represented a more-slowly-turning-over tissue zinc pool.

The values for the rate constants (ie, $k_{ij}$) and their uncertainties were ascertained from the model at the least-squares fit. The steady-state solution of the model, calculated by assuming a known value (mg) for $Q_1$ and by assuming that all entry and irreversible loss of zinc occurred through the plasma compartment, provided estimates (in mg) of $Q_2$ and $Q_3$, as well as estimates (in mg/h) of all mass fluxes ($R_{ij}$) between compartments.

The $Q_1$ mass was calculated by multiplying the estimated total plasma volume by the measured plasma zinc concentration. Plasma volume (mL) was calculated from body surface area (m$^2$) multiplied by 1440, with the 50% predicted increase in plasma volume during pregnancy being taken into account (18). We assumed that the plasma volume increased by 50% from EP to L.P. The exchangeable zinc pool, calculated as the sum of $Q_1$, $Q_2$, and $Q_3$, represented the total space in which the isotopic tracer equilibrated in 24 h.

**Statistical analysis**

Statistical analysis was performed by using STATGRAPHICS software (version 7; Manugistics, Cambridge, MA). Longitudinal comparisons were done by using repeated-measures analysis of variance. Pairwise significant differences were assessed by using Tukey’s range test. Associations between variables were examined by simple correlation analysis. Values were considered significant at $P < 0.05$.

**RESULTS**

The women were healthy, had appropriate weight-for-height, and had a normal blood hemoglobin concentration in EP (Table 1). All women had normal length of gestation (37–42 wk) except one woman, who had a premature delivery at 31 wk gestation. Gestational weight gains were between 6.5 and 18.9 kg. The

![Figure 1. Three-compartment model of 24-h zinc kinetics, in which the circles represent kinetically distinct zinc compartments. $Q_1$ represents the plasma pool, and $Q_2$ and $Q_3$ represent a rapidly-turning-over and a slowly-turning-over tissue zinc pool, respectively.](image)

**Table 1. Characteristics of the women studied**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>Prepregnancy BMI (kg/m$^2$)</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>Parity</td>
<td>2.6 ± 1.6</td>
</tr>
<tr>
<td>Early-pregnancy blood hemoglobin (g/L)</td>
<td>121 ± 10</td>
</tr>
<tr>
<td>Length of gestation (wk)</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>Weight gain during gestation (kg)</td>
<td>11.4 ± 5.8</td>
</tr>
<tr>
<td>Infant birth weight (kg)</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>Infant birth length (cm)</td>
<td>51 ± 2</td>
</tr>
</tbody>
</table>

*All values are $\bar{x} \pm$ SD; n = 9 (the 10th woman in the study delivered prematurely; see text).*
women gave birth to healthy infants who weighed 2.1 kg (premature delivery) and from 2.7 to 4.4 kg (term deliveries) at birth and who gained weight normally during the lactation period. The women habitually consumed diets that had marginal zinc content (Table 2). Over the entire study, mean dietary zinc intake was 8.9 mg/d, and mean molar phytate:zinc was 17.4. Beans, rice, corn meal, and manioc flour were the major sources of dietary phytate. Dietary intakes of energy, protein, zinc, iron, fiber, and phytate did not change significantly during the study.

Plasma zinc concentrations decreased 25% from EP to LP (P < 0.01) and returned to the EP concentration at EL (Table 3). Erythrocyte zinc was significantly higher at LP and EL than at EP (P < 0.02). Erythrocyte metallothionein did not change significantly during the study.

Urinary zinc did not change significantly during pregnancy and lactation (Table 3). FZA was 49% and 37% higher at LP and EL, respectively, than at EP (P < 0.03). FZA was inversely related to plasma zinc concentrations at LP, but the relation was not significant (r = −0.718, P = 0.069). A significant inverse relation was evident at EL (r = −0.732, P = 0.016) (Figure 2).

The kinetic values measured varied significantly between subjects (Table 4). There were no significant changes during the study in any of the rate constants, but some trends were apparent. Compared with EP, the rate of zinc movement at LP from Q1 to Q2 (k2,1) tended to be higher, whereas that from Q1 to Q3 (k3,1) tended to be lower. In contrast, at EL, the rate of zinc movement from Q1 to Q2 tended to be lower and that from Q1 to Q3 tended to be higher than the same movements at EP.

The Q1 mass increased ≈23% from EP to LP (P < 0.05) and returned to the EP mass at EL (Table 4). There were no significant changes in the mass in the other compartments. However, compared with EP, the trend at LP was toward a greater mass in Q2 and a smaller mass in Q3, and the trend at EL was toward a smaller mass in Q2 and a greater mass in Q3.

The exchangeable zinc pool (the sum of Q1, Q2, and Q3) did not change significantly during pregnancy and lactation. Zinc flux from plasma (Q1) to the most-rapidly-turning-over extravascular pool (Q2) (ie, R2,1) increased ≈53% from EP to LP (P < 0.05) (Table 4). Zinc flux from plasma (Q1) into the less-rapidly-turning-over pool (Q3) (ie, R3,1) increased ≈27% from EP to EL, although this increase was not significant. These changes represent the movement of an additional 75 mg Zn/d from

**TABLE 2**
Dietary intakes from 3-d weighed-food records during pregnancy and lactation

<table>
<thead>
<tr>
<th></th>
<th>EP</th>
<th>LP</th>
<th>EL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal/d)</td>
<td>1910 ± 660</td>
<td>2130 ± 400</td>
<td>2190 ± 610</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>69 ± 30</td>
<td>79 ± 26</td>
<td>70 ± 21</td>
</tr>
<tr>
<td>Zinc (mg/d)</td>
<td>9 ± 5</td>
<td>9 ± 2</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>Iron (mg/d)</td>
<td>13 ± 6</td>
<td>14 ± 5</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>Phytate (mg/d)</td>
<td>1290 ± 660</td>
<td>1680 ± 840</td>
<td>1770 ± 930</td>
</tr>
<tr>
<td>Molar phytate:zinc</td>
<td>16 ± 8</td>
<td>19 ± 7</td>
<td>18 ± 9</td>
</tr>
<tr>
<td>Dietary fiber (g/d)</td>
<td>20 ± 9</td>
<td>23 ± 8</td>
<td>20 ± 6</td>
</tr>
</tbody>
</table>

1 All values are ± SD; EP, early pregnancy (10–12 wk); LP, late pregnancy (34–36 wk); EL, early lactation (7–8 wk).

**TABLE 3**
Biochemical zinc indexes, urinary zinc excretion, and fractional zinc absorption (FZA) during pregnancy and lactation

<table>
<thead>
<tr>
<th></th>
<th>EP (n = 10)</th>
<th>LP (n = 9)</th>
<th>EL (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma zinc (μmol/L)</td>
<td>11.4 ± 1.7</td>
<td>8.5 ± 0.7</td>
<td>11.2 ± 3.5</td>
</tr>
<tr>
<td>Erythrocyte zinc (μmol/g protein)</td>
<td>0.50 ± 0.07</td>
<td>0.54 ± 0.05</td>
<td>0.57 ± 0.11</td>
</tr>
<tr>
<td>Erythrocyte metallothionein (nmol/g protein)</td>
<td>2.9 ± 0.6</td>
<td>2.9 ± 0.5</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>Urinary zinc (μmol/d)</td>
<td>8.4 ± 3.1</td>
<td>9.5 ± 3.5</td>
<td>8.6 ± 4.3</td>
</tr>
<tr>
<td>FZA 2</td>
<td>0.29 ± 0.06</td>
<td>0.43 ± 0.10</td>
<td>0.39 ± 0.13</td>
</tr>
</tbody>
</table>

1 All values are ± SD; EP, early pregnancy (10–12 wk); LP, late pregnancy (34–36 wk); EL, early lactation (7–8 wk).

2 Because of analytic problems with 1 subject at LP, longitudinal comparison of FZA involved only 8 subjects.

**FIGURE 2.** Relation by simple correlation analysis between fractional zinc absorption and plasma zinc at early lactation (n = 10) (r = −0.73, P = 0.02). The regression equation is fractional zinc absorption (%) = 0.702 − 0.028 plasma zinc (μmol/L).

**TABLE 4**
Zinc kinetic parameters of the 3-compartment model during pregnancy and lactation

<table>
<thead>
<tr>
<th></th>
<th>EP (n = 10)</th>
<th>LP (n = 9)</th>
<th>EL (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate constant (h⁻¹)</td>
<td>k2,1 1.0 ± 0.5</td>
<td>0.9 ± 0.5</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>k3,1 3.4 ± 1.2</td>
<td>4.4 ± 2.3</td>
<td>3.1 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>k3,1 4.1 ± 0.9</td>
<td>2.0 ± 1.5</td>
<td>3.7 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>k3,1 3.6 ± 1.2</td>
<td>3.1 ± 1.7</td>
<td>4.4 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>k3,1 0.12 ± 0.06</td>
<td>0.2 ± 0.3</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td>Pool size (mg)</td>
<td>Q1 1.7 ± 0.3</td>
<td>2.1 ± 0.3</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Q2 4.9 ± 2.7</td>
<td>5.8 ± 2.6</td>
<td>3.8 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>Q3 45 ± 13</td>
<td>41 ± 19</td>
<td>52 ± 22</td>
</tr>
<tr>
<td></td>
<td>EZP (mg) 52 ± 13</td>
<td>49 ± 19</td>
<td>58 ± 23</td>
</tr>
<tr>
<td>Flux (mg/h)</td>
<td>R0,1 1.6 ± 0.8</td>
<td>1.7 ± 0.7</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>R2,1 5.7 ± 1.7</td>
<td>8.7 ± 3.3</td>
<td>5.6 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>R3,1 6.2 ± 2.8</td>
<td>6.0 ± 2.4</td>
<td>7.9 ± 3.0</td>
</tr>
</tbody>
</table>

1 All values are ± SD; EP, early pregnancy (10–12 wk); LP, late pregnancy (34–36 wk); EL, early lactation (7–8 wk); Q1, plasma zinc pool; Q2, a rapidly-turning-over tissue zinc pool; Q3, a more-slowly-turning-over tissue zinc pool; EZP, exchangeable zinc pool (the sum of Q1, Q2, and Q3). Means in a row with different superscript letters are significantly different, P < 0.05 (repeated-measures analysis of variance and Tukey’s test).
plasma into $Q_2$ at LP and of an additional 40 mg Zn/d from plasma into $Q_3$ at EL.

**DISCUSSION**

The adaptation of zinc homeostasis to pregnancy and lactation in women who habitually consume marginal zinc diets has been addressed in cross-sectional studies (4–7). To our knowledge, this is the first longitudinal study of intestinal zinc absorption and zinc kinetics during pregnancy and lactation in women with marginal dietary zinc intake ($=9$ mg/d) and a moderate molar phytate:zinc ($<20$). The women did not change their habitual dietary intake during the study, which made it possible to study adjustments of zinc metabolism during pregnancy and lactation, when diet was essentially constant.

Although zinc intake was marginal, changes in plasma and erythrocyte zinc during pregnancy and lactation in the study subjects were similar to those described elsewhere in women with higher zinc intakes (1, 8, 19), which indicated normal physiologic zinc adjustments to these variables. Erythrocyte metallothionein concentrations in these subjects were similar to those measured previously at delivery in women with higher zinc intakes (15), probably in response to the high concentrations of estrogen and progesterone during pregnancy (20).

Cross-sectional studies indicate that the efficiency of zinc absorption may be high during pregnancy and lactation in women with diets that are low in zinc (4, 6, 7). In the current study, FZA measured from a standardized breakfast meal was 0.29 at EP; it increased $=49\%$ at LP and remained high at EL. These longitudinal results, taken together with results from previous cross-sectional studies done at lactation (6, 7), indicated that an increased efficiency of zinc absorption at LP and during lactation contributes to meeting the increased zinc needs during reproduction, when zinc intakes are marginal.

The pattern of change in FZA during pregnancy and lactation in the women in the current study who were consuming $=9$ mg Zn/d was similar to that in the women who were consuming $=15$ mg Zn/d (8); there were higher rates of absorption at LP and EL, when the need for zinc for reproduction is high. The rates of absorption were significantly higher among the women with lower usual zinc intakes than among the women with higher usual zinc intakes, eg, 43% and 19% at LP and 39% and 25% at EL, respectively (Table 5). These differences suggest that the efficiency of zinc absorption may be greater in pregnant and lactating women with low zinc intakes. However, differences between the amount of zinc fed in the test meals in the current study and in that of Fung et al (8) may account for the different rates of zinc absorption. Total zinc intake inversely affects the efficiency of the absorption of zinc from a test meal (21). The total zinc intake in the test meal in the current study was only 30% of that in the study of Fung et al (8). The lower amount of zinc in the test meal in the current study probably contributed to the higher efficiencies of absorption in the Brazilian women. The increase in efficiency was insufficient, however, to equalize the amount of zinc absorbed from the test meal in our study and that of Fung et al: total absorbed zinc was about 35% lower at LP and 55% lower at EL in the women in the current study than in those in the study by Fung et al. Further studies are needed to establish the independent effects of dietary zinc intake and maternal zinc status on the homeostatic adjustments in zinc absorption during pregnancy and lactation.

Iron supplementation during pregnancy and lactation may have an effect on zinc absorption. In cross-sectional studies done during pregnancy (4) and lactation (22), zinc absorption was significantly lower in iron-supplemented women than in non-supplemented women. In a longitudinal study from before pregnancy to lactation (8), FZA did not increase significantly in women who took iron supplements during lactation. In all of these studies, the iron supplement was consumed during periods of measurement of zinc absorption, which favored gastrointestinal interaction between the iron supplement and the zinc tracer. In the current study, however, the women stopped taking the iron supplement during the clinical trial to reduce the potential interaction of iron with dietary and tracer zinc in the gastrointestinal tract. We previously showed that supplementation with iron (100 mg/d) as ferrous sulfate taken separately from meals for 8 wk did not affect the absorption of zinc from a test meal in nonpregnant women (23). However, the effect on zinc absorption and homeostasis of iron supplementation and iron status during pregnancy and lactation should be investigated further.

The zinc status of the woman appears to be an important determinant of the physiologic adjustments made to meet zinc demands during pregnancy and lactation (3). In the women in the current study, FZA was inversely but not significantly related to plasma zinc concentrations at LP and at EL. It is possible that lower maternal plasma zinc concentrations somehow signaled the intestinal mucosa to increase the efficiency of zinc absorption when zinc needs were elevated. The lower plasma zinc concentrations at LP (8.5 and 9.8 μmol/L) and EL (11.2 and 12.5

<table>
<thead>
<tr>
<th>Study</th>
<th>Test meal</th>
<th>Oral isotope</th>
<th>Total</th>
<th>Before pregnancy or early pregnancy</th>
<th>Late pregnancy</th>
<th>Early lactation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
<td>% mg</td>
<td>% mg</td>
<td>% mg</td>
</tr>
<tr>
<td>Fung et al (8)</td>
<td>1.22$^i$</td>
<td>3.0</td>
<td>4.22</td>
<td>14.6$^i$</td>
<td>0.62</td>
<td>19.4</td>
</tr>
<tr>
<td>Current study</td>
<td>0.22</td>
<td>1.0</td>
<td>1.22</td>
<td>28.7$^i$</td>
<td>0.35</td>
<td>42.8</td>
</tr>
</tbody>
</table>

$^i$ EB Fung, personal communication, September 2003.

$^j$ Before pregnancy.

$^k$ Early pregnancy.
μmol/L) in the women in the current study than in those studied by Fung et al, respectively, are consistent with this hypothesis. Experimental human zinc-depletion studies have shown that FZA increases when zinc intakes, and possibly zinc status, are reduced (24). An inverse relation between intestinal zinc absorption and maternal plasma zinc concentrations may contribute to improved fetal growth and breast milk synthesis and secretion when maternal diets are marginal in zinc. This may explain, in part, why little or no association between maternal plasma zinc and infant birth weight has been observed in women of low socioeconomic status (25). Urinary zinc excretion did not increase during pregnancy in the women in the current study, whereas it did increase in other studies (1, 8). It is possible that renal zinc conservation contributed to zinc homeostasis in women consuming low-zinc diets. The addition of supplemental zinc to iron and folate supplements increased urinary zinc concentrations at LP in Peruvian women who were consuming ≈7 mg Zn/d (26). The role of renal function in zinc homeostasis during pregnancy and lactation should be investigated further.

Compartmental analysis of zinc tracer kinetics has been used to describe zinc metabolism in men (27, 28) and in nonpregnant, nonlactating women (11). We also used a short-term zinc kinetics model to describe the effect of low zinc intakes in pregnant rats (29). In that study, we found that the turnover rate of the exchangeable plasma zinc pool increased with marginal intakes. On the basis of this experience with zinc compartmental modeling in pregnant rats, we felt that zinc kinetic studies could provide valuable insights into zinc homeostasis among women who had marginal zinc intakes.

In the current study, a 3-compartment model was used to describe the sizes and turnover rates of 2 extravascular pools (Q2 and Q3) that exchanged with the plasma zinc pool (Q1) over a 24-h period. The turnover time of compartment Q3 was <1 h, and that of compartment Q2 was <10 h. Previous studies in men (30, 31), nonpregnant women (32), and pregnant rats (29) suggested that Q2 and Q3 are located within the liver, erythrocytes, and kidney, and, when the subject is pregnant, they may include the fetus. Similar anatomical studies have not been done in lactating animals.

Although the rate constants between zinc pools did not change significantly during pregnancy and lactation in this study, there tended to be an increased rate of plasma transfer into Q2 at LP and into Q3 during lactation. The high demands for zinc during pregnancy and lactation appear to increase the rate at which zinc moves from plasma into rapidly-turning-over zinc pools, but the flux goes to a different pool during pregnancy than during lactation. At LP, there was a 53% increase in zinc flux from plasma into Q2, as a result of the combined increase in the rate constant k2,1 and of the Q1 mass. On the basis of this increased flux in LP, we estimate that an additional 75 mg Zn/d was transferred into the rapidly-turning-over zinc pool, which possibly reflected the increased zinc demands of the fetus and of zinc metabolism in maternal tissues, such as liver and bone marrow, during LP. The zinc flux from the plasma into compartment Q1 during EL was 27% greater than that during EP; this greater flux contributed an additional transfer of 40 mg Zn/d from plasma into this pool, which may include the mammary gland and may, therefore, increase the availability of zinc for milk synthesis.

In summary, the plasma zinc flux between 2 exchangeable pools in LP differed significantly from that in EL. But the size of the total exchangeable zinc pool and the irreversible flux of zinc out of the system (R0,1) into very-slowly-turning-over zinc pools or as zinc excretory losses were unchanged during pregnancy and lactation. To meet the increased demand for zinc during LP, the increased transfer of 75 mg Zn/d from the plasma into a rapidly-turning-over zinc pool was augmented by a 49% increase in the efficiency of intestinal zinc absorption. During lactation, the efficiency of zinc absorption increased by 37%, and zinc transfer from the plasma to a less-rapidly-turning-over exchangeable zinc pool increased by 40 mg/d (which was not significant). Women with lower plasma zinc concentrations had higher efficiencies of zinc absorption in EL. These results show that the homeostatic adjustments in zinc metabolism to meeting the demands of fetal growth differ from those needed to meet the demands of milk synthesis.

We thank the medical and nursing staffs of Maternidade Escola of the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, for their valuable support during the clinical part of the study. We are very grateful to all the women who participated in this study.

CMD was responsible for the supervision of the study and writing of the manuscript and was involved in study design and data analysis. CLVZ was responsible for the clinical study and for data collection and analysis. LWR participated in management and laboratory analysis. DMS and RM conducted the estimates of fractional zinc absorption and zinc kinetics. JCK was responsible for the conception and funding of the study and was involved in study design and writing of the manuscript. All authors reviewed the manuscript. None of the authors had a personal or financial conflict of interest.

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Effects of maternal docosahexaenoic acid intake on visual function and neurodevelopment in breastfed term infants

Craig L Jensen, Robert G Voigt, Thomas C Prager, Yali L Zou, J Kennard Fraley, Judith C Rozelle, Marie R Turcich, Antolin M Llorente, Robert E Anderson, and William C Heird

ABSTRACT

Background: Normal brain and visual development is thought to require exogenous docosahexaenoic acid (DHA; 22:6n–3) intake, but the amount needed is debatable. Because the supplementation of breastfeeding mothers with DHA increases the DHA content of their infants’ plasma lipids, we hypothesized that it might also improve brain or visual function in the infants.

Objective: The objective was to determine the effect of DHA supplementation of breastfeeding mothers on neurodevelopmental status and visual function in the recipient infant.

Design: Breastfeeding women received capsules containing either a high-DHA algal oil (~200 mg DHA/d) or a vegetable oil (no DHA) for 4 mo after delivery. Outcome variables included the fatty acid pattern of maternal plasma phospholipids and milk lipids 4 mo postpartum, the fatty acid pattern of plasma phospholipids and visual function in infants at 4 and 8 mo of age, and neurodevelopmental indexes of the infants at 12 and 30 mo of age.

Results: Milk lipid and infant plasma phospholipid DHA contents of the supplemented and control groups were ~75% and ~35% higher, respectively, at 4 mo postpartum. However, neither the neurodevelopmental indexes of the infants at 12 mo of age nor the visual function at 4 or 8 mo of age differed significantly between groups. In contrast, the Bayley Psychomotor Development Index, but not the Mental Development Index, of the supplemented group was higher (P < 0.01) at 30 mo of age.

Conclusion: DHA supplementation of breastfeeding mothers results in higher infant plasma phospholipid DHA contents during supplementation and a higher Bayley Psychomotor Development Index at 30 mo of age but results in no other advantages either at or before this age.

KEY WORDS Maternal docosahexaenoic acid supplementation, infant visual function, infant neurodevelopmental indexes, fatty acid pattern of milk lipid, infant plasma phospholipid fatty acid pattern, Bayley Scales of Infant Development

INTRODUCTION

Most studies of the effects of docosahexaenoic acid (DHA; 22:6n–3) on infant development have focused on the effect of supplementing formula-fed infants with DHA and how closely the development of supplemented infants mimics the development of breastfed infants. However, the amount of DHA in human milk is highly variable (1, 2), being low in some US women, particularly in comparison with the milk of women from regions where fish consumption is high (1–12). Thus, studies of breastfed infants receiving different DHA intakes should also be helpful in determining the role of DHA intake during early infancy on subsequent visual function and neurodevelopment.

We (13) and others (14–16) have shown that the supplementation of breastfeeding women with DHA increases the DHA content of their plasma lipids and milk as well as that of their infant’s plasma lipids. Because the DHA content of plasma lipids is thought to reflect the DHA content of brain and retina, which, in turn, is important for normal brain and visual function, we hypothesized that DHA supplementation of breastfeeding mothers would increase the DHA content of the recipient infants’ plasma lipids and, perhaps, improve visual and neuropsychological development in the recipient infants. The results of our attempt to test this hypothesis are reported here.

SUBJECTS AND METHODS

Subjects

Pregnant women who planned to breastfeed exclusively for ≥4 mo were recruited by newspaper ads, flyers in physicians’ offices, and presentations at childbirth classes. Inclusion criteria included an age between 18 and 40 y, infant gestational age ≥37 wk, and infant birth weight between 2500 and 4200 g. Exclusion

1 From the US Department of Agriculture/Agricultural Research Service Children’s Nutrition Research Center (CLJ, JKF, and WCH) and the Meyer Center for Developmental Pediatrics (RGV, JCR, MRT, and AML), Department of Pediatrics, Baylor College of Medicine, Houston, TX; the Hermann Eye Center, University of Texas Health Science Center, Houston, TX (TCP and YLZ); and the Dean A McGee Eye Institute, University of Oklahoma Health Sciences Center, Oklahoma City, OK (REA).

2 This work is a publication of the US Department of Agriculture/Agricultural Research Service Children’s Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, TX. The contents of this publication do not necessarily reflect the view or policies of the US Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

3 Supported by grants from the US Department of Agriculture/National Research Initiative (9700693); Martek Biosciences Corp, Columbia, MD; and Mead-Johnson Nutritional, Evansville, IN.

4 Address reprint requests to CL Jensen, Children’s Nutrition Research Center, Baylor College of Medicine, 1100 Bates Street, Houston, TX 77030. E-mail: cjensen@bcm.tmc.edu.

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criteria included chronic maternal disorders as well as major congenital anomalies and obvious gastrointestinal or metabolic disorders of the infant. The study was approved by the Institutional Review Board for Human Subject Research of Baylor College of Medicine and Affiliated Hospitals and the Committee for the Protection of Human Subjects of the University of Texas Health Sciences Center at Houston. Written informed consent was obtained from all mothers before enrollment.

Study design

Women who qualified were assigned randomly in a double-masked manner, with the use of a computer-generated randomization scheme, to receive 1 of 2 identical capsules daily for 4 mo, starting within 5 d after delivery. One capsule contained a high-DHA algal triacylglycerol (DHASCO; Martek Biosciences Corp, Columbia, MD) that consisted of 44% saturated fatty acids, 13.6% monounsaturated fatty acids, 0.8% linoleic acid (18:2n-6), and 41.7% DHA (22:6n-3) by weight; it provided ~200 mg DHA/d. The control capsule contained a 50:50 mixture of soy and corn oils consisting of 15% saturated fatty acids, 23.5% monounsaturated fatty acids, 56.3% linoleic acid (18:2n-6), and 3.9% α-linolenic acid (18:3n-3). Both capsules were supplied at no cost by Martek Biosciences Corp. They were dispensed at each visit before 4 mo postpartum by the Investigational Pharmacy of Texas Children’s Hospital according to the previously mentioned randomization scheme. The number of capsules dispensed was always more than needed before the next appointment at wk 2, mo, or 4 mo postpartum; the subjects were instructed to ingest one capsule per day and to return all unused capsules at the next appointment. On the basis of the number of capsules returned, women in both groups took from 95% to 100% of the capsules they were instructed to take.

The primary outcome variable was performance at 30 mo of age on the Bayley Scales of Infant Development (17). Other outcome variables included the fatty acid pattern of milk lipids at 4 mo postpartum as well as that of infant plasma phospholipids at 4 and 8 mo postpartum, infant visual function at 4 and 8 mo of age, and additional indexes of neurodevelopmental status at 12 and 30 mo of age. Infant weight, length, and head circumference were monitored throughout the study.

Milk and blood collections

Mothers and infants were admitted to the Metabolic Research Unit of the Children’s Nutrition Research Center at 4 mo postpartum for a 24-h milk collection. At each feeding, the infant was offered one breast, and an electrical breast pump (Egnell Inc, Cary, IL) was used to empty the contents of the other breast with each subsequent feeding. A fixed percentage of each volume collected was obtained, mixed thoroughly, added to similar collections during the 24-h collection period, and frozen at −70 °C for subsequent analysis. Blood samples from mothers and infants were obtained by venipuncture. Plasma was separated by centrifugation (2450 × g, 10 min, room temperature) and frozen at −70 °C until analyzed.

Plasma phospholipid and milk fatty acid measurements

Plasma and milk lipids were extracted by the method of Bligh and Dyer (18), and the phospholipid fraction of plasma was separated by one-dimensional thin-layer chromatography (Silica Gel 60; Sigma-Aldrich, St Louis, MO), as described previously (19). Methyl esters of the component fatty acids of the milk lipid and plasma phospholipid fractions were prepared with boron trifluoride methanol and quantified, as described previously (19, 20), by gas chromatography (Varian 3500; Varian Inc, Palo Alto, CA) with a DB-225 capillary column (J & W Scientific, Folsom, CA). The content of each fatty acid was expressed as the mole percentage of total fatty acids.

Assessment of visual function

Binocular visual acuity was assessed at 4 and 8 mo of age by using the Teller Acuity Card procedure as well as both sweep and transient visual evoked potentials (VEPs). The details of all methods were described previously (21). Briefly, the Teller Acuity Card procedure takes advantage of the fact that infants prefer to look at a patterned rather than an unpatterned stimulus. Cards with a grating pattern on one side and no pattern on the other side are presented to an infant by an examiner, who observes the infant through a peephole in the center of the card and determines the direction in which the infant looks without knowledge of the direction of the pattern. Cards with gratings of increasing spatial frequency (ie, smaller stripes) are presented until the infant is judged to be unable to discriminate the location of the pattern. Visual acuity is determined by the highest spatial frequency judged by the examiner to be distinguishable by the infant.

Transient and sweep VEPs were performed by using the ENFANT 4010 Electrophysiology System (NeuroScientific Corporation, Farmingdale, NY). At both ages, the infants were seated on a parent’s lap, 75 cm from the monitor. During each session an investigator observed the direction of the child’s gaze and either paused or resumed recording if necessary. Another investigator verified that the corneal reflection of the stimulus was aligned with the pupil and, to maximize fixation, jiggled a set of metal keys in the center of the screen when the child appeared inattentive.

Transient VEPs were recorded with the use of pattern reversals of checkerboard stimuli. VEP latency (the time, in milliseconds, between the presentation of the stimulus and the peak of the occipital cortex response) and amplitude (the magnitude, in μV, of the recorded occipital cortex response) were determined. Sweep VEPs, performed by measuring the amplitude of the electrical response of the occipital cortex as visual stimuli (square wave gratings, or bars), were presented rapidly; the size of the bar gratings were “swept” from low to high spatial frequencies in 0.56 cycles/degree steps ranging from 0.56 to 6.75 cycles/degree. These stimuli have sharp edges and, hence, approximate Snellen eye chart acuity more closely than do simple sinusoidal gratings. Regression software provided by the instrument manufacturer was used to calculate the estimated threshold of resolution (ie, spatial frequency corresponding to a VEP amplitude of 0 μV).

Assessment of neurodevelopmental status

The primary outcome variable was the score on the Bayley Scales of Infant Development at 30 mo of age (17). These scales, considered the gold standard for the assessment of global abilities of children aged <3 y, assess overall mental development, including language and visual-motor problem solving [Mental Development Index (MDI)] and both fine and gross motor development [Psychomotor Development Index (PDI)]. The instrument was administered by 1 of 2 psychologists trained and
experienced in its use. It was administered at 30 mo of age because assessments at this age are more predictive of later function than are assessments at earlier ages (22).

Gross motor development of the infants was assessed at 12 and 30 mo of age by using the gross motor scale of the Gesell Developmental Inventory (23). At the same ages, language development was assessed by the Clinical Linguistic and Auditory Milestone Scale (CLAMS), and visual-motor problem-solving abilities were assessed by the Clinical Adaptive Test (CAT) (24, 25). All of these tests were administered by the same developmental pediatrician, and the results were expressed as developmental quotients (DQ). These tests were chosen because they can be administered much more quickly than can the Bayley Scales. We have shown that the overall CAT/CLAMS DQ (the mean of the CLAMS DQ and the CAT DQ) is correlated with concurrent Bayley MDI scores at both 12 (r = 0.393, P = 0.008) and 30 (r = 0.742, P = 0.0001) mo of age and that the overall CAT/CLAMS DQ at 12 mo of age is correlated modestly with the Bayley MDI at 30 mo of age (r = 0.181, P = 0.036) (26).

Anthropometric measures

Nude weights of the infants were determined with an electronic integrating scale (model LC 16000s; Sartorius, Gottingen, Germany) at 21 d as well as at 2, 4, 8, 12, 18, 24, and 30 mo of age. Length and head circumference were measured at the same times by using an infant stadiometer (Holtain Ltd, Crymlyn, United Kingdom) and a metal measuring tape (Executive Thinline 2 m; Lufkin Tape, Apex, NC), respectively.

Data analysis

Data are expressed as group means ± SDs. The statistical significance of differences in continuous outcome variables between groups was tested by independent-samples t tests (SPSS software; SPSS, Chicago, IL).

Group differences in neurodevelopmental outcomes were further evaluated by analysis of covariance with control for sex, ethnicity, maternal age, maternal education, maternal intelligence quotient (as determined with the Slosson Intelligence Test, revised—a test that screens for the intellectual abilities of adults; 27), the composite score of the Family Environment Scale (28), birth weight, duration of breastfeeding, weight, and body length at the time each test was administered. Correlations between selected outcome variables were determined by regression analysis. The statistical significance of differences in categorical variables between groups was tested by the chi-square statistic or Fisher’s exact test. A probability of ≤5% was assumed to represent statistical significance.

The number of mother-infant pairs enrolled was selected to permit detection of a difference in the Bayley MDI or PDI between groups at 30 mo of age of ≥0.5 SD (power = 80%; P < 0.05), assuming a completion rate through 30 mo of ≈60% (i.e., an anticipated dropout rate of ≈40% through 30 mo of age). Averaging the data from twin pairs (1 in the DHA group and 2 in the control group) and treating the pair as a single subject compared with treating each twin as an individual subject did not affect any outcome variables. The data for each set of twins were reported as data for an individual subject.

RESULTS

Subjects

One hundred fourteen mothers were assigned to the DHA group and 113 to the control group. Each group included 115 infants (one group of twins in the DHA group; 2 in the control group). The number of subjects lost during the study and the number of subjects available for study at 4, 12, and 30 mo of age are summarized in Figure 1. Most subject losses occurred before the 4-mo assessment, primarily because breastfeeding was stopped or the intake of foods other than breast milk exceeded 20% of intake—a preset exclusion criterion; other losses occurred because the mothers decided to discontinue participation (3 in DHA group; 4 in control group) or to relocate (2 in DHA group; 0 in control group). All subject losses after 4 mo resulted because the family had relocated (2 in the DHA group between 4 and 12 mo of age and 5 in the DHA group between 12 and 30 mo; 4 in the control group between 4 and 12 mo of age and 6 in the control group between 12 and 30 mo). Thus, 90 and 87 infants in the DHA and control groups, respectively, were available for study at 12 mo and 83 and 77, respectively, were available for study at 30 mo.

The 2 groups of mothers did not differ significantly by age at delivery (31.5 ± 5 y in both groups), parity (1.8 ± 1.1 and 1.7 ± 0.9 in the DHA and control groups, respectively), or years of education (15.9 ± 2.2 and 16.3 ± 2.7 in the supplemented and control groups, respectively). The demographic characteristics of the 2 groups of infants (including 1 set of twins in the DHA group and 2 sets in the control group) also did not differ significantly at enrollment (Table 1) and the demographic characteristics of those remaining in the study at 4, 8, 12, and 30 mo of age did not differ significantly from those of the total group enrolled.

Milk and infant plasma fatty acids

Mean mole percentages of selected n–3 and n–6 fatty acids in milk lipid at 4 mo postpartum and in infant plasma phospholipids at 4 mo of age are shown in Tables 2 and 3, respectively. The DHA content of milk lipids was significantly greater in the DHA group than in the control group (P < 0.0001), and the contents of arachidonic acid (20:4n–6) and 22:4n–6 (docosatetraenoic acid) were lower (P < 0.001 and < 0.004, respectively). Infant plasma phospholipid fatty acid pattern at 4 mo of age mirrored that of milk lipid. The DHA content was significantly higher in the infants whose mothers were assigned to the DHA group than in those in the control group (P < 0.0001), and the contents of 20:4n–6, 22:4n–6, and 22:5n–6 (osbond acid) were significantly lower (P < 0.001, <0.001, and < 0.001, respectively). There were no statistically significant differences in infant plasma phospholipid fatty acid patterns between groups at 8 mo of age (data not shown).

Visual function

Visual acuity as assessed by the Teller Acuity Card procedure at 4 and 8 mo of age and by Sweep VEP at 4 mo of age, both expressed as cycles/degree ± octave, is shown in Table 4. There were no significant differences between groups in either measure at 4 or 8 mo of age. Acuity, as assessed by Sweep VEP at 8 mo of age, was 10.3 ± 0.24 and 10.6 ± 0.19 cycles/degree in the DHA and control groups, respectively. We have no explanation
for why these values were lower than expected at this age, particularly because Teller acuities at this age are within the expected range (30).

Mean Transient VEP latency and amplitude at 4 and 8 mo of age at a check size of 30’ are shown in Table 5. There was no statistically significant difference in transient VEP latency between groups at either age. However, at both 4 and 8 mo of age, the transient VEP amplitude of the group whose mothers were assigned to the DHA group was significantly lower than that of the group whose mothers were assigned to the control group.

Neurodevelopmental status

There were no statistically significant differences between groups in the Gesell Gross Motor Inventory, CAT, or CLAMS DQ at either 12 (Table 6) or 30 mo of age (Table 7). The Bayley MDI of the 2 groups at 30 mo of age, one of the primary outcome variables, also did not differ significantly, but the other primary outcome variable—the Bayley PDI at 30 mo of age—was 8.4 points higher (P = 0.005; independent samples t-test) in the group whose mothers received DHA. This difference remained

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**FIGURE 1.** Number of subjects enrolled, lost, and tested over the 30-mo study period. The docosahexaenoic acid (DHA) group included 1 set of twins, and the control group included 2 sets of twins; thus, the number of infants in each group was identical (n = 115).
TABLE 1
Demographic characteristics of infants whose mothers were assigned to receive algal docosahexaenoic acid (DHA) or a control treatment for 4 mo postpartum.

<table>
<thead>
<tr>
<th></th>
<th>Algal DHA group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 115) (^1)</td>
<td>(n = 115) (^1)</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>3.46 ± 0.55(^2)</td>
<td>3.48 ± 0.51</td>
</tr>
<tr>
<td>Gestational age at birth (wk)</td>
<td>39.4 ± 1.4</td>
<td>39.5 ± 1.3</td>
</tr>
<tr>
<td>Appar score at 1 min</td>
<td>8.3 ± 1.0</td>
<td>8.0 ± 1.7</td>
</tr>
<tr>
<td>Appar score at 5 min</td>
<td>9.0 ± 0.3</td>
<td>8.8 ± 1.4</td>
</tr>
<tr>
<td>Sex (% boys)</td>
<td>57</td>
<td>60</td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>75</td>
<td>79</td>
</tr>
<tr>
<td>African American</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>Hispanic</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cesarean delivery (%)</td>
<td>34</td>
<td>29</td>
</tr>
</tbody>
</table>

\(^1\) The DHA group included 1 set of twins, and the control group included 2 sets of twins; each set of twins was treated as an individual infant (see Data analysis).

\(^2\) ± SD (all such values).

statistically significant (P = 0.008) after infant sex, ethnicity, birth weight, duration of breastfeeding, weight and length at 30 mo of age, maternal age, maternal education, maternal IQ (27), and the composite score of the Family Environment Scale (28) were controlled for..

Three subjects whose mothers received the control capsule and one whose mother received the DHA-containing capsule had PDI scores <75. However, eliminating these subjects from the analysis, while reducing the SD of both groups, did not affect the statistical significance of the difference between groups; thus, the data presented include the scores of all subjects because we know of no reason for exclusion. Subjects whose mothers received DHA accounted for 64%, 49%, and 38% of the upper, middle, and lower tertiles of PDI scores, respectively; whereas subjects whose mothers received the control oil accounted for 36%, 51%, and 62% of the respective tertiles. There was no statistically significant correlation between infant plasma phospholipid DHA content at either 4 or 8 mo of age and any measure of visual function or neurodevelopment.

TABLE 2
Fatty acid composition of milk lipids at 4 mo postpartum.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Algal DHA group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 83)</td>
<td>(n = 77)</td>
</tr>
<tr>
<td>18:3n−3</td>
<td>1.20 ± 0.90</td>
<td>1.07 ± 0.35</td>
</tr>
<tr>
<td>20:5n−3</td>
<td>0.07 ± 0.04</td>
<td>0.07 ± 0.08</td>
</tr>
<tr>
<td>22:5n−3</td>
<td>0.12 ± 0.04</td>
<td>0.14 ± 0.08</td>
</tr>
<tr>
<td>22:6n−3</td>
<td>0.35 ± 0.14</td>
<td>0.20 ± 0.24(^2)</td>
</tr>
<tr>
<td>18:2n−6</td>
<td>16.3 ± 2.8</td>
<td>15.9 ± 3.6</td>
</tr>
<tr>
<td>20:4n−6</td>
<td>0.40 ± 0.08</td>
<td>0.44 ± 0.08(^3)</td>
</tr>
<tr>
<td>22:4n−6</td>
<td>0.08 ± 0.02</td>
<td>0.09 ± 0.02(^4)</td>
</tr>
<tr>
<td>22:5n−6</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.02</td>
</tr>
</tbody>
</table>

\(^1\) All values are ± SD. Data were not available for 6 participants in the docosahexaenoic acid (DHA) group and 8 in the control group.

\(^2\)−\(^4\) Significantly different from the algal DHA group (independent-samples t test): \(^2\) P < 0.0001, \(^3\) P < 0.001, \(^4\) P < 0.004.

Anthropometric measures

There were no statistically significant differences in weight, length, or head circumference between the 2 groups of infants at any time. At all ages, these measures were within the normal range for age in both groups.

DISCUSSION

The study reported here is one of only a few that has addressed the effects of supplementing breastfeeding mothers with DHA on visual function and subsequent neuropsychological development of the recipient infant. In this study, maternal supplementation with ~200 mg DHA/d for 4 mo after delivery resulted in a 50% greater content of maternal plasma phospholipid DHA (data not shown), an 75% greater content of DHA in milk lipids, and an 35% greater content of DHA in infant plasma phospholipids. However, the higher infant plasma phospholipid DHA content was not accompanied by detectable effects on visual acuity as measured by the Teller Acuity Card procedure or

TABLE 3
Fatty acid composition of the phospholipid fraction of infant plasma at 4 mo postpartum.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Algal DHA group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 80)</td>
<td>(n = 79)</td>
</tr>
<tr>
<td>% of total fatty acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3n−3</td>
<td>0.18 ± 0.06</td>
<td>0.21 ± 0.13</td>
</tr>
<tr>
<td>20:5n−3</td>
<td>0.29 ± 0.16</td>
<td>0.30 ± 0.16</td>
</tr>
<tr>
<td>22:5n−3</td>
<td>0.54 ± 0.12</td>
<td>0.76 ± 0.21</td>
</tr>
<tr>
<td>22:6n−3</td>
<td>4.81 ± 1.12</td>
<td>3.57 ± 1.08(^5)</td>
</tr>
<tr>
<td>18:2n−6</td>
<td>21.7 ± 2.6</td>
<td>21.5 ± 2.9</td>
</tr>
<tr>
<td>20:4n−6</td>
<td>10.1 ± 2.0</td>
<td>11.4 ± 2.4(^3)</td>
</tr>
<tr>
<td>22:4n−6</td>
<td>0.37 ± 0.09</td>
<td>0.46 ± 0.12(^2)</td>
</tr>
<tr>
<td>22:5n−6</td>
<td>0.35 ± 0.12</td>
<td>0.49 ± 0.16(^2)</td>
</tr>
</tbody>
</table>

\(^5\) All values are ± SD. Data were not available for 10 infants whose mothers were assigned to the docosahexaenoic acid (DHA) group and 8 whose mothers were assigned to the control group.

\(^2\)−\(^3\) Significantly different from the algal DHA group (independent-samples t test): \(^2\) P < 0.0001, \(^3\) P < 0.001.

TABLE 4
Visual acuity of the 2 groups of infants at 4 and 8 mo of age as measured by the Teller Acuity Card procedure and at 4 mo of age as measured by sweep visual evoked potential (VEP).

<table>
<thead>
<tr>
<th></th>
<th>Algal DHA group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cycles/degree</td>
<td></td>
</tr>
<tr>
<td>Teller Acuity Card procedure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 mo</td>
<td>5.6 ± 0.71([70])</td>
<td>5.3 ± 0.56([77])</td>
</tr>
<tr>
<td>8 mo</td>
<td>12.3 ± 0.53([74])</td>
<td>13.5 ± 0.57([73])</td>
</tr>
<tr>
<td>Sweep VEP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 mo</td>
<td>9.4 ± 0.23([81])</td>
<td>9.4 ± 0.21([79])</td>
</tr>
</tbody>
</table>

\(^1\) The variance is expressed as octaves: the SD of the log-transformed values divided by 0.301 (29). Statistical analysis (independent-samples t test) was done after logarithmic transformation of raw acuity scores. Cycles/degree values are the antilog of the mean of the logarithms of individual raw cycles/degrees of each group at each time. There were no statistically significant differences in Teller acuity between groups at either age or in sweep VEP between groups at 4 mo of age (independent-samples t test).
CLAMS DQ 100.6
/L50512
CAT DQ 109.0
Gesell Gross Motor DQ 101.8

DHA did not have a higher Gesell Gross Motor, CAT, or CLAMS and visual acuity in the present study.

The correlation between infant plasma phospholipid DHA content findings of other studies, there was no statistically significant
tent of DHA and visual acuity (33, 34). In contrast with the
dressed the effect of the natural variation in breast-milk DHA
showed a statistically significant positive correlation between
neurontic as well as toxic insults early in life have been reported
mental or the nature of the developmental tests. For example,
t is somewhat puzzling, it could result from early supple-
ence of an early nutritional intervention, such as DHA, may not be
absence of obvious visual advantages at 4 and 8 mo of age, developmental advantages at 12 mo of age, or other advantages at
30 mo of age, is difficult to assess. On one hand, the difference
between groups was sizable and remained statistically significant
after many potential covariates were controlled for. On the
other hand, the PDI at 30 mo of age was not correlated with
plasma phospholipid DHA at either 4 or 8 mo of age. Furthermore, although the PDI at 30 mo of age is a better predictor of
development at later than at earlier ages (22), the magnitude of
this advantage is not clear, particularly because the mean PDI of
both groups was well above the expected mean. Considering
these facts, it is tempting to attribute the higher PDI of the sup-plemented group at 30 mo of age to chance.

Sweep VEP at either 4 or 8 mo of age. The transient VEP am-
plitude of the DHA-supplemented group was lower than that of
the control group at both ages, but the biologic significance of
this finding is not clear. Although a greater occipital cortex re-
ponse to a visual stimulus suggests more optimal neural pro-
cessing, transient VEP amplitude of normal infants actually de-
clines with age, primarily because of increasing skull thickness
(21).

Most other randomized controlled studies of this issue have
also shown no differences in visual acuity between control in-
fants and infants whose mothers were assigned randomly to
receive even higher amounts of DHA or fish oil during pregnancy
or during early lactation (15, 31, 32). However, most of these
showed a statistically significant positive correlation between
visual acuity and the DHA content of infant plasma or erythro-
cyte phospholipids at the same or earlier age. Studies that ad-
ressed the effect of the natural variation in breast-milk DHA
content have also shown a statistically significant positive cor-
relation between infant plasma or erythrocyte phospholipid con-
tent of DHA and visual acuity (33, 34). In contrast with the
findings of other studies, there was no statistically significant
correlation between infant plasma phospholipid DHA content
and visual acuity in the present study.

Although children whose mothers were supplemented with
DHA did not have a higher Gesell Gross Motor, CAT, or CLAMS
DQ at either 12 or 30 mo of age, those whose mothers received
DHA had an 8.4-point (＞0.5 SD) higher Bayley Scales of Infant
Development PDI at 30 mo of age than did those who received
placebo. The meaning of this higher PDI, particularly in the

**TABLE 5**

<table>
<thead>
<tr>
<th>Age (mo)</th>
<th>Latency (ms)</th>
<th>Amplitude (μV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>124.8 ± 11.7 [86]</td>
<td>28.9 ± 12.1 [86]</td>
</tr>
<tr>
<td>8</td>
<td>115.1 ± 8.1 [79]</td>
<td>24.3 ± 8.9 [79]</td>
</tr>
</tbody>
</table>

1 All values are x ± SD; n values in brackets. DHA, docosahexaenoic acid.
2 Significantly different from the algal DHA group, P < 0.03 (independent-samples t test).

**TABLE 6**

| Neurodevelopmental outcomes of the 2 groups of children at 12 mo of age
<table>
<thead>
<tr>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Algal DHA group</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Gesell Gross Motor DQ</td>
</tr>
<tr>
<td>CAT DQ</td>
</tr>
<tr>
<td>CLAMS DQ</td>
</tr>
</tbody>
</table>

1 All values are x ± SD. Because of scheduling difficulties, data were not available for 2 infants whose mothers were assigned to the docosahexaenoic acid (DHA)–supplemented group and for 7 infants whose mothers were assigned to the control group. DQ, developmental quotient; CAT, Clinical Adaptive Test; CLAMS, Clinical Linguistic and Auditory Milestone Scale. There were no statistically significant differences between groups for any measure.

**TABLE 7**

| Neurodevelopmental outcomes of the 2 groups of children at 30 mo of age
<table>
<thead>
<tr>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Algal DHA group</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Gesell Gross Motor DQ</td>
</tr>
<tr>
<td>CAT DQ</td>
</tr>
<tr>
<td>CLAMS DQ</td>
</tr>
<tr>
<td>Bayley MDI</td>
</tr>
<tr>
<td>Bayley PDI</td>
</tr>
</tbody>
</table>

1 All values are x ± SD; n value in brackets. Eighty-three children whose mothers were assigned to the DHA–supplemented group and 77 whose mothers were assigned to the control group were available for testing, but only the numbers of children indicated could be scheduled within the designated age window (30 ± 3 mo). One child in the algal DHA group successfully completed the Bayley PDI but not the MDI, whereas one child in the control group successfully completed the Bayley MDI but not the PDI. DHA, docosahexaenoic acid; DQ, developmental quotient; CAT, Clinical Adaptive Test; CLAMS, Clinical Linguistic and Auditory Milestone Scale; MDI, Mental Development Index; PDI, Psychomotor Development Index.  
2 Significantly different from the algal DHA group, P = 0.008 [analysis of covariance with control for sex, ethnicity, maternal age, maternal education, maternal intelligence quotient (27), the composite score of the Family Environment Scale (28), birth weight, duration of breastfeeding, and weight and length at the time each test was administered].
apparent until later in life. A beneficial effect of maternal cod liver oil compared with corn oil supplementation (10 mL/d), from 18 wk of gestation through 3 mo postpartum, on the cognitive function of infants at 4 y of age has also been reported (38). In this study, children whose mothers received cod liver oil (>1200 mg DHA and >800 mg 20:5n-3/d) scored >4 points higher at 4 y of age on the Mental Processing Composite of the Kaufman Assessment Battery for Children (K-ABC). However, only 34 children of the 590 pregnant women enrolled in the study completed the K-ABC at 4 y of age.

A potential relation between DHA status and neuropsychological function, even in adulthood, is supported by recent studies that suggest that low serum cholesteryl ester DHA (39) and low dietary DHA intakes (40) are associated with a higher risk of Alzheimer disease. Later effects of early DHA supplementation on cardiovascular function also have been reported (41); children who were supplemented with DHA for the first 4 mo of life had significantly lower mean and diastolic blood pressures at 6 y of age than did children who were not supplemented.

The higher 30-mo PDI in infants whose mothers received DHA rather than the control oil, for only the first 4 mo postpartum, suggests that infants of breastfeeding women whose dietary n-3 fatty acid intake is low may benefit from maternal DHA supplementation. This might also suggest that formula-fed infants may benefit from a formula with a DHA content in excess of 0.2% of total fatty acids. However, specific recommendations along these lines should await confirmation of the findings reported here in either breastfed or formula-fed infants. Although the apparent benefits of maternal DHA supplementation and, hence, a higher DHA intake by the infant on the Bayley PDI at 30 mo of age was sizeable, and possibly explicable, additional studies are required to determine whether this apparent advantage is real, whether this advantage persists, and whether other delayed effects of early DHA availability emerge.

We gratefully acknowledge the assistance of C Boutte (Study Coordinator) and Rebecca Newsom (Administrative Secretary) and thank the children and parents who participated in the study.

CLJ, JKF, and WCH had full access to all data and take responsibility for the integrity and accuracy of the data and for the data analysis. CLJ, WCH, RGV, TCP, and REA conceived and designed the study. CLJ, RGV, TCP, YLZ, JCR, MRT, and WCH acquired the data. CLJ, JKF, WCH, RGV, TCP, and AML analyzed and interpreted the data. CLJ and WCH drafted the manuscript. RGV, TCP, YLZ, JCR, MRT, AML, and REA critically revised the manuscript for important intellectual content. JKF provided statistical analysis and AML analyzed and interpreted the data. CLJ and WCH drafted the manuscript for important intellectual content. JKF provided statistical analysis and AML analyzed and interpreted the data. CLJ and WCH drafted the manuscript for important intellectual content.

REFERENCES


Biochemical indexes of the B vitamins in cord serum are predicted by maternal B vitamin status\textsuperscript{1,2}

Rima Obeid, Winfried Munz, Monika Jäger, Werner Schmidt, and Wolfgang Herrmann

ABSTRACT
Background: The concentration of total homocysteine (tHcy) is higher in newborns than in older children. Vitamin B-12 is the major determinant of tHcy in newborns. Maternal status of folate, vitamin B-12, and vitamin B-6 during pregnancy may affect the biochemical markers of these micronutrients in newborns.

Objective: Our objective was to study the relation between concentrations of the metabolites and B vitamins in maternal sera and concentrations in the umbilical venous blood of the corresponding newborns.

Design: We studied healthy pregnant women at the time of labor who were expecting healthy, full-term, appropriate-birth-weight babies. Samples were available from 82 mother-infant pairs.

Results: Concentrations of B vitamins were higher in cord samples than in maternal blood (folate, 2-fold; vitamin B-12, 1.5-fold; and vitamin B-6, 6-fold). Concentrations of cystathionine and methylmalonic acid (MMA) were also higher in the infants than in the mothers (\( \bar{x} \pm SD \): cystathionine, 462 \( \pm \) 189 and 343 \( \pm \) 143 nmol/L; MMA, 353 \( \pm \) 144 and 233 \( \pm \) 110 nmol/L). No significant differences in tHcy concentrations were observed between fetal and maternal samples. Concentrations of vitamin B-12 did not differ significantly between mothers of infants from different quartiles of cord MMA. Higher fetal MMA concentrations were related to higher maternal status of folate, vitamin B-12, and vitamin B-6, and tHcy was interrelated, and the tHcy concentration is a sensitive indicator of the status of the B vitamins. Moreover, the concentration of methylmalonic acid (MMA) becomes specifically elevated in the blood of vitamin B-12-deficient persons (10). Cystathionine is an intermediate product of homocysteine transsulfuration. This compound increases in the case of vitamin B-6 deficiency. The combined use of the metabolic markers (tHcy, MMA, and cystathionine) improves the sensitivity and the specificity of detecting possible disturbances in the remethylation and the transsulfuration pathways (10).

Conclusions: Maternal concentrations of the metabolic markers of B vitamins predict values in fetal blood at delivery. Fetal tHcy concentrations were low but were predicted by the vitamin status of the mother. The effect of increasing maternal intake of vitamins B-12 and B-6 during pregnancy on the fetal concentrations of the metabolites should be investigated.

KEY WORDS  Homocysteine, methylmalonic acid, cystathionine, folate, vitamin B-12

INTRODUCTION
The requirements for B vitamins (folate, vitamin B-12, and vitamin B-6) are exceptionally high during pregnancy as a result of increased maternal metabolic rate and fetal demands (1). Available evidence suggests that maternal nutritional status before and during pregnancy is the main determinant of the nutritional status of the offspring (2, 3). Folate, vitamin B-12, and vitamin B-6 function as cofactors in one-carbon metabolism, DNA synthesis, and numerous methylation reactions. These metabolic pathways are particularly active in developing embryos.

Poor maternal status of B vitamins has been linked to pregnancy complications and poor outcomes (4–8). B vitamins may lower serum concentrations of total homocysteine (tHcy) (3) and may protect against undesirable pregnancy outcomes (7, 9). The metabolism of folate, vitamin B-12, vitamin B-6, and tHcy is interrelated, and the tHcy concentration is a sensitive indicator of the status of the B vitamins. Moreover, the concentration of methylmalonic acid (MMA) becomes specifically elevated in the blood of vitamin B-12-deficient persons (10). Cystathionine is an intermediate product of homocysteine transsulfuration. This compound increases in the case of vitamin B-6 deficiency. The combined use of the metabolic markers (tHcy, MMA, and cystathionine) improves the sensitivity and the specificity of detecting possible disturbances in the remethylation and the transsulfuration pathways (10).

Several studies investigated B vitamin status in pregnant women and their newborns (11–15). Maternal concentrations of tHcy before delivery were strongly related to preconception levels and, more importantly, to tHcy concentrations in umbilical cord blood (3, 11). Folate-supplemented mothers and their newborns had lower concentrations of tHcy at the time of labor than did those who had not taken supplements (3). Studies of neonates (aged 3 d to 6 mo) have shown markedly higher serum concentrations of tHcy and MMA in these infants than in older children (16). Moreover, concentrations of tHcy and MMA were higher in newborns 6 wk after birth than at birth (2). However, most neonates remain virtually asymptomatic. Elevated concentrations of tHcy in neonates are related to higher concentrations of MMA, cystathionine, or methionine (16, 17). In contrast with the case in older children, the metabolic changes were associated with lower concentrations of vitamin B-12 rather than folate (16). Therefore, a transient inadequate vitamin B-12 status and a disturbed transmethylation at this age have been suspected (2, 16, 17).
reports mentioned above prompted us to examine the concentration of the metabolites and the corresponding vitamins in umbilical venous blood at birth. The present study was designed to investigate the relation between the concentration of the metabolites and B vitamins in maternal sera and in the venous umbilical cord sera of the corresponding newborns.

SUBJECTS AND METHODS

Subjects and clinical data

Pregnant women admitted with the onset of confirmed labor were randomly recruited among consecutive deliveries at the Department of Obstetrics and Gynecology, University Hospital of Saarland. Inclusion criteria were being a healthy pregnant woman aged ≥17 y and free of chronic diseases or pregnancy complications and expecting a singleton, full-term (>37 wk), healthy infant of appropriate length and weight. Both vaginal and cesarean births were included. Samples from 82 mothers and from the venous umbilical cord blood of their newborns were available at the end of the study.

Gestational age was defined on the basis of information about the last menstrual period and an ultrasound estimation of the fetus size. This was then confirmed after birth by a physical examination of the newborn. Maternal anthropologic measures and data on smoking, diet, vitamin use during pregnancy, parity, and gravidity were obtained by interviewing the participants on admission. Parity was defined as primiparous (no former children) or multiparous (parity of 1 or ≥2). Clinical characteristics of the newborns were also documented (weight, length, head circumference, blood gases, and venous and arterial blood pH). The study was approved by the Medical Ethical Committee of the University Hospital of Saarland, and all participants gave their informed consent to participate.

Blood sampling and laboratory analysis

Nonfasting blood samples were obtained from the antecubital vein of the mothers 1–12 h before birth. Immediately after delivery, the cord was clamped at both ends and cut. A blood sample was collected from the umbilical vein. Maternal and cord blood samples were collected in tubes without anticoagulant, left to clot, and centrifuged within 45 min at 2000 × g and 4 °C. Serum was directly separated and stored at −70 °C until analyzed.

Serum concentrations of tHcy, cystathionine, and MMA were measured by using gas chromatography–mass spectrometry as described elsewhere (18). Maternal and cord blood were measured in the same run. Concentrations of vitamin B-12 and folate were measured by using a chemiluminescence immunoassay (ADVIA Centaur System, Bayer, Germany). The serum concentration of vitamin B-6 (pyridoxal-5'-phosphate, or PLP) was determined by HPLC with a fluorescence detector and reagents from Immundiagnostik (Bensheim, Germany). The reference values in our laboratory for nonpregnant women (aged 17–42 y) are as follows: >207 pmol/L for vitamin B-12, >16.8 nmol/L for folate, and >36.1 nmol/L for vitamin B-6 (the 5th percentiles), <13.0 μmol/L for tHcy, <243 nmol/L for MMA, and <290 nmol/L for cystathionine (the 95th percentiles).

Statistical analysis

The distribution of each continuous variable was assessed by using the Kolmogorov-Smirnov test. All variables were skewed and were therefore log-transformed to approach a normal distribution before tests were applied that propose such a distribution of the data. Data are presented as means or geometric means and SDs. Means of continuous variables were compared between mothers and cord sera by using the paired Student’s t test. Intragroup multiple comparisons were performed by using one-way analysis of variance (ANOVA). The post hoc Tamhane test was performed to identify significantly different group means when the ANOVA test was significant. Multivariable backward regression analyses were conducted to identify significant variables that predicted changes in the concentrations of the metabolites in cord sera. Correlations between different variables were examined by using Spearman’s test. All tests were two-sided, and P values < 0.05 were considered to be statistically significant.

RESULTS

The main characteristics of the participating women and their newborns are shown in Table 1. Concentrations of some biochemical markers in maternal and cord serum samples are shown in Table 2. Significantly higher concentrations of MMA, folate, vitamin B-12, and PLP were observed in cord serum samples than in maternal sera (Table 2). Mean concentrations of folate, vitamin B-12, and PLP were 2-, 1.5-, and 6-fold higher, respectively, in fetal than in maternal sera. Mean concentrations of cystathionine were slightly lower in the mothers than in cord blood samples. No significant differences in the concentrations of tHcy were found between maternal and cord sera (Table 2). The interindividual CVs for fetal tHcy, cystathionine, and MMA were 34%, 40%, and 39%, respectively. The CVs of maternal concentrations were 27% for Hcy, 38% for cystathionine, and 43% for MMA.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mothers (n = 82)</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>30 ± 6'</td>
</tr>
<tr>
<td>Current BMI (kg/m²)</td>
<td>28.2 ± 4.4</td>
</tr>
<tr>
<td>Weight increase during pregnancy (kg)</td>
<td>12.7 ± 5.0</td>
</tr>
<tr>
<td>Vaginal delivery [n (%)]</td>
<td>56 (68)</td>
</tr>
<tr>
<td>Smoking [n (%)]</td>
<td>18 (22)</td>
</tr>
<tr>
<td>Folate supplement during pregnancy [n (%)]</td>
<td>14 (17)</td>
</tr>
<tr>
<td>Parity [n (%)]</td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>36 (44)</td>
</tr>
<tr>
<td>Parity of 1</td>
<td>27 (33)</td>
</tr>
<tr>
<td>Parity ≥ 2</td>
<td>19 (23)</td>
</tr>
<tr>
<td>Infants (n = 82)</td>
<td></td>
</tr>
<tr>
<td>Gestational age (wk)</td>
<td>39.5 ± 1.1</td>
</tr>
<tr>
<td>Boys [n (%)]</td>
<td>42 (51)</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3363 ± 423</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>35 ± 1.8</td>
</tr>
<tr>
<td>Venous blood pH</td>
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</tr>
<tr>
<td>Arterial blood pH</td>
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</tr>
<tr>
<td>P&lt;CO₂ (mm Hg)²</td>
<td>45.0 ± 9.1</td>
</tr>
<tr>
<td>P&lt; O2 (mm Hg)²</td>
<td>20.2 ± 15.2</td>
</tr>
</tbody>
</table>

1 ± SD (all such values).
2 P<CO₂ partial pressure of carbon dioxide; P< O2 partial pressure of oxygen.

TABLE 1

Main characteristics of the mothers and infants

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</tr>
<tr>
<td>P&lt; O2 (mm Hg)²</td>
<td>20.2 ± 15.2</td>
</tr>
</tbody>
</table>
TABLE 2
Concentrations of the metabolites and B vitamins in maternal serum and in cord blood samples.

| Serum marker | Mothers (n = 82) | Cord blood (n = 82) | Fetal-maternal difference | Fetal/maternal ratio | P
|--------------|----------------|-------------------|--------------------------|---------------------|---------
| tHcy (μmol/L) | 5.62 ± 1.61 | 5.37 ± 1.93 | −0.12 ± 1.50 | 0.96 ± 0.30 | 0.484
| Cystathionine (nmol/L) | 343 ± 143 | 462 ± 189 | 214 ± 969 | 1.35 ± 0.39 | 0.052
| MMA (nmol/L) | 233 ± 110 | 353 ± 144 | 119 ± 122 | 1.51 ± 0.57 | <0.001
| Vitamin B-12 (pmol/mL) | 172 ± 48 | 248 ± 128 | 86 ± 81 | 1.44 ± 0.48 | <0.001
| PLP (nmol/mL) | 13.1 ± 9.4 | 78.0 ± 72.9 | 82.0 ± 67.9 | 6.04 ± 3.57 | <0.001
| Folate (nmol/mL) | 27.0 ± 19.6 | 60.9 ± 21.1 | 30.7 ± 15.0 | 2.23 ± 1.19 | <0.001

1 tHcy, total homocysteine; MMA, methylmalonic acid; PLP, pyridoxal-5’-phosphate.
2 Geometric x ± SD.
3 Calculated as 100 × (SD/x).
4 x ± SD.
5 Paired Student’s t test.

Some important correlations between maternal and cord blood markers are shown in Table 3. Gestational age correlated significantly with the concentration of cystathionine in cord serum (r = −0.33, P = 0.004). This correlation remained significant after adjustment for fetal PLP or maternal cystathionine. Maternal concentrations of MMA and vitamin B-12 did not correlate significantly (r = −0.16, P = 0.148).

Concentrations of B vitamins and their metabolites in cord and maternal sera according to quartiles of MMA in cord serum are shown in Table 4. A higher concentration of cord MMA was associated with higher concentrations of tHcy and cystathionine in cord blood. The mean concentration of fetal vitamin B-12 was significantly higher in infants with lower MMA than in those with higher MMA. Furthermore, mean concentrations of MMA were significantly higher in mothers of infants in the fourth quartile of cord MMA than in mothers of infants in the first MMA quartile. Concentrations of vitamin B-12 did not differ significantly between mothers of infants from different quartiles of cord MMA.

The differences between fetal and maternal MMA (ΔMMA) increased along quartiles of fetal MMA. On the other hand, differences in vitamin B-12 between maternal and cord sera (ΔB-12) were higher in the lowest quartile of cord MMA (Table 4). Concentrations of MMA in cord sera showed a negative and highly significant correlation with ΔB-12 (Figure 1). Backward regression analysis showed that concentrations of MMA in cord sera were predicted by maternal MMA (β = 0.565, P < 0.001), maternal vitamin B-12 (β = 0.379, P = 0.012), and fetal vitamin B-12 (β = −0.362, P = 0.001).

Shown in Figure 2 are the mean (95% CI) concentrations of folate, PLP, tHcy, and cystathionine in maternal and cord sera according to quartiles of tHcy in cord serum. Mothers of infants with higher concentrations of tHcy had lower concentrations of folate and PLP and higher concentrations of tHcy than did mothers of infants with lower concentrations of tHcy. Higher concentrations of tHcy in cord serum were also associated with slightly lower concentrations of cord folate and PLP and higher concentrations of tHcy than did mothers of infants with lower concentrations of tHcy. Higher concentrations of tHcy in cord serum were also associated with slightly lower concentrations of cord folate and PLP and higher concentrations of tHcy than did mothers of infants with lower concentrations of tHcy. Higher concentrations of tHcy in cord serum were also associated with slightly lower concentrations of cord folate and PLP and higher concentrations of tHcy than did mothers of infants with lower concentrations of tHcy. Higher concentrations of tHcy in cord serum were also associated with slightly lower concentrations of cord folate and PLP and higher concentrations of tHcy than did mothers of infants with lower concentrations of tHcy. Higher concentrations of tHcy in cord serum were also associated with slightly lower concentrations of cord folate and PLP and higher concentrations of tHcy than did mothers of infants with lower concentrations of tHcy. Higher concentrations of tHcy in cord serum were also associated with slightly lower concentrations of cord folate and PLP and higher concentrations of tHcy than did mothers of infants with lower concentrations of tHcy.

Shown in Figure 3 are the mean (95% CI) maternal and fetal concentrations of cystathionine, tHcy, folate, and PLP according to concentrations of cystathionine in cord serum. Higher concentrations of cystathionine in cord serum were associated with lower fetal and maternal PLP, higher maternal cystathionine, and higher fetal and maternal tHcy. Regression analysis showed that gestational age (β = −2.881, P = 0.009), maternal cystathionine (β = 0.574, P < 0.001), fetal tHcy (β = 0.234, P = 0.014), and fetal PLP (β = −0.113, P = 0.015) were significant factors that predicted cystathionine concentrations in cord serum.

TABLE 3
Correlations between maternal serum and cord blood markers.

<table>
<thead>
<tr>
<th>Maternal markers</th>
<th>Cord blood markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>tHcy</td>
<td>Cystathionine</td>
</tr>
<tr>
<td>Gestational age</td>
<td>−0.13</td>
</tr>
<tr>
<td>tHcy</td>
<td>0.60</td>
</tr>
<tr>
<td>Cystathionine</td>
<td>0.42</td>
</tr>
<tr>
<td>MMA</td>
<td>0.06</td>
</tr>
<tr>
<td>Folate</td>
<td>−0.28</td>
</tr>
<tr>
<td>Vitamin B-12</td>
<td>0.04</td>
</tr>
<tr>
<td>Vitamin B-6</td>
<td>−0.23</td>
</tr>
</tbody>
</table>

1 Correlations were assessed by Spearman’s ρh test. tHcy, total homocysteine; MMA, methylmalonic acid.
2 P < 0.05.
Both the correlation coefficient and vitamin B-12 concentrations between cord and maternal sera (tions of methylmalonic acid (MMA) in cord serum and the difference in mothers who reported having taken folate supplementation during boys and those of girls (data not shown). As would be expected, from girls. Comparable values were also found in mothers of in cord blood

Concentrations of some biochemical markers in cord blood and in maternal serum according to quartile (Q) of methylmalonic acid (MMA) concentrations in cord blood

<table>
<thead>
<tr>
<th></th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
<th>$P^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cord blood markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMA (nmol/L)</td>
<td>250 ± 37</td>
<td>308 ± 13</td>
<td>362 ± 19</td>
<td>558 ± 150</td>
<td>—</td>
</tr>
<tr>
<td>Vitamin B-12 (pmol/L)</td>
<td>298 ± 79</td>
<td>281 ± 211</td>
<td>232 ± 69$^a$</td>
<td>194 ± 68$^a$</td>
<td>0.002</td>
</tr>
<tr>
<td>tHcy (μmol/L)</td>
<td>4.81 ± 1.28</td>
<td>5.07 ± 1.94</td>
<td>5.44 ± 1.68</td>
<td>6.35 ± 2.40$^a$</td>
<td>0.031</td>
</tr>
<tr>
<td>Folate (nmol/L)</td>
<td>62.4 ± 26.8</td>
<td>62.1 ± 19.1</td>
<td>61.7 ± 18.1</td>
<td>57.1 ± 19.9</td>
<td>0.796</td>
</tr>
<tr>
<td>Cystathionine (nmol/L)</td>
<td>426 ± 182</td>
<td>378 ± 163</td>
<td>488 ± 217</td>
<td>588 ± 2030</td>
<td>0.045</td>
</tr>
<tr>
<td><strong>Maternal markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMA (nmol/L)</td>
<td>168 ± 47</td>
<td>221 ± 76$^a$</td>
<td>247 ± 80$^a$</td>
<td>323 ± 144$^a$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin B-12 (pmol/L)</td>
<td>179 ± 50</td>
<td>181 ± 57</td>
<td>169 ± 35</td>
<td>159 ± 49</td>
<td>0.379</td>
</tr>
<tr>
<td>tHcy (μmol/L)</td>
<td>5.43 ± 1.63</td>
<td>5.63 ± 1.40</td>
<td>5.63 ± 1.42</td>
<td>5.81 ± 1.98</td>
<td>0.572</td>
</tr>
<tr>
<td>Folate (nmol/L)</td>
<td>24.9 ± 20.4</td>
<td>26.8 ± 14.0</td>
<td>26.4 ± 18.0</td>
<td>30.6 ± 25.0</td>
<td>0.769</td>
</tr>
<tr>
<td>Cystathionine (nmol/L)</td>
<td>358 ± 145</td>
<td>330 ± 104</td>
<td>361 ± 204</td>
<td>324 ± 101</td>
<td>0.162</td>
</tr>
<tr>
<td><strong>Fetal-maternal difference</strong>$^3$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>∆MMA (nmol/L)</td>
<td>79 ± 41</td>
<td>75 ± 74</td>
<td>104 ± 82</td>
<td>223 ± 185</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>∆B-12 (pmol/L)</td>
<td>126 ± 66</td>
<td>106 ± 112</td>
<td>72 ± 67</td>
<td>39 ± 46</td>
<td>0.002</td>
</tr>
<tr>
<td>∆tHcy (μmol/L)</td>
<td>0.59 ± 1.65</td>
<td>0.31 ± 1.42</td>
<td>0.04 ± 1.27</td>
<td>0.51 ± 1.53</td>
<td>0.125</td>
</tr>
<tr>
<td>∆Folate (nmol/L)</td>
<td>35.2 ± 19.8</td>
<td>31.4 ± 8.7</td>
<td>33.2 ± 12.9</td>
<td>22.5 ± 13.9</td>
<td>0.034</td>
</tr>
</tbody>
</table>

$^1$ tHcy, total homocysteine; ∆, difference.
$^2$ Geometric $\bar{x}$ ± SD.
$^3$ Geometric $\bar{x}$ ± SD.
$^4$ Significantly different from Q1, $P < 0.05$ (post hoc Tamhane test).
$^5$ $\bar{x}$ ± SD.

**Other important analyses**

Serum concentrations of all biochemical markers did not differ significantly between cord serum samples from boys and those from girls. Comparable values were also found in mothers of boys and those of girls (data not shown). As would be expected, mothers who reported having taken folate supplementation during pregnancy ($n = 14$) had significantly higher concentrations of serum folate than did unsupplemented mothers (median: 35.5 compared with 22.9 nmol/L; $P = 0.026$). Folate was also slightly higher in infants of supplemented mother than in infants of unsupplemented mothers (median: 73.2 compared with 58.0; $P = 0.064$).

Eighteen mothers who smoked during pregnancy reported having smoked <10 cigarettes daily. Significantly lower serum concentrations of folate were detected in smoking than in nonsmoking mothers (median: 18.4 compared with 30.6 nmol/L; $P = 0.009$). Moreover, lower cord serum concentrations of folate were observed in cord blood samples from newborns of smoking mothers than in samples from newborns of nonsmoking mothers (median: 57.1 compared with 61.0; $P = 0.039$). These findings were not related to folate supplementation during pregnancy because smoking mothers and their infants differed from nonsmoking mothers and their infants when only non-vitamin-users were considered in the analysis.

Neither the type of delivery nor the duration of the vaginal delivery was related to any of the markers investigated in this study (data not shown). No relation between parity and the vitamins or the metabolites could be confirmed in this study even after omitting the mothers who took folate during pregnancy.

**DISCUSSION**

Maternal vitamin deficiency during pregnancy may have significant implications on the progress and outcome of the pregnancy. Insufficient maternal vitamin B-12 status may affect methylation potential in neonates (19). The current study included a healthy group of pregnant women and their newborn infants. Concentrations of B vitamins and their metabolites showed a relatively wide range of interindividual variations. The most remarkable finding in this study was that markers of the B vitamins (folate, vitamin B-12, and vitamin B-6) at birth were related to maternal markers.

![FIGURE 1. Scatter plot representing the correlation between concentrations of methylmalonic acid (MMA) in cord serum and the difference in vitamin B-12 concentrations between cord and maternal sera (∆B-12). The correlation coefficient and $P$ value are according to Spearman’s test. Data on both the $x$ and the $y$ axes are anti-log.](image-url)
Concentration of B vitamins in cord serum in relation to maternal serum

The concentration of B vitamins was considerably higher in fetal serum than in maternal serum (Table 2). A marked decrease in the serum concentration of B vitamins is known to take place during pregnancy (1, 20–25). Loading pregnant women with pyridoxal HCl was followed by a prompt increase in serum concentrations of PLP (24). Most importantly, higher concentrations of PLP were detected in cord serum after vitamin B-6 loading of the mothers than in cord serum from unsupplemented mothers (24). Furthermore, the PLP concentration showed an arterial-venous gradient with higher concentrations in umbilical venous than in arterial serum (26). These findings indicate that vitamin B-6 is readily transformed into its active form and then actively transferred to the fetus.

Data about the transplacental transfer of B vitamins are limited. It was reported that the placenta synthesizes transcobalamin and is rich with transcobalamin receptor (27). Furthermore, vitamin B-12 and folate are efficiently sequestered in the intervillous space of the placenta (28). The placenta was shown to accumulate vitamin B-12 injected in pregnant animals (29). Therefore, the placenta seems to extract these essential micronutrients from the maternal circulation and redistribute them, probably favoring the fetus (26, 29–32). Rappazzo et al (33) investigated vitamin B-12 content in fetal and adult tissues. Despite high concentrations of vitamin B-12 in fetal serum, the fetal liver contained only 30% of the adult liver vitamin B-12 content calculated as pg vitamin B-12/mg tissue. The authors concluded that the fetus retains most of the vitamin B-12 in the blood and utilizes the available amount for biochemical reactions (33). The wide range of between-subjects CVs for fetal vitamin B-12 (49% in the cord samples compared with 27% in the mothers) also suggests that the transport of maternal vitamin B-12 may be determined by factors other than the concentrations of the vitamin.

Concentration of the metabolites in maternal and cord sera

The concentration of tHcy in cord samples did not differ significantly from that of the mothers (Table 2). Previous studies reported lower tHcy concentrations in cord serum than in maternal serum (3, 11). However, the mean maternal tHcy concentration was lower in our study than in previous ones (3, 11, 19). Additionally, differences in study designs, timing of blood collection, analytic methods, or the groups studied may be responsible for these different findings. As in previous reports (3), the concentration of tHcy in cord and maternal sera was strongly correlated in our study (Table 3). Previous studies showed that concentrations of methionine are much higher in cord than in maternal blood samples (19, 34). On the other hand, fetal concentrations of tHcy seemed to be maintained at low levels, despite the fact that the fetus is likely to be exposed to high concentrations of methionine. The high requirement for methyl groups implies that the transmethylation of methionine is active in developing embryos. Therefore, tHcy can be formed from methionine in the fetus. Moreover, tHcy was lower in the umbilical artery than in the umbilical vein (11). These results strongly suggest that tHcy remethylation to methionine is highly active during prenatal life because tHcy concentrations are relatively low in cord blood. This is probably due to the high fetal
demand for tetrahydrofolate in DNA synthesis. Cystathionine is also formed as a product of tHcy catabolism (discussed below).

Concentrations of MMA were higher in cord blood than in the mothers or in healthy adults (Table 2; 10, 19). Concentrations of MMA were predicted by cord vitamin B-12 and maternal MMA (Table 2, 3). In addition, the regression analysis showed that higher maternal vitamin B-12 was a predictor of higher fetal MMA. Moreover, lower cord MMA was associated with larger differences between cord blood and maternal concentrations of vitamin B-12, but not with higher maternal concentrations of vitamin B-12 (Figure 1, Tables 3 and 4). These results suggest a rate-limiting step in the transplacental transport of vitamin B-12. Polymorphisms in the transcobalamin gene are one factor that might influence the transport of vitamin B-12 from the placenta to the fetus. Moreover, high serum concentrations of vitamin B-12 in cord blood may be related to a slower uptake of the vitamin, probably because of a lower expression or activity of transcobalamin receptor. A slow rate of MMA elimination from the plasma compartments in the fetus may also be related to higher MMA at birth. Finally, the positive association between fetal concentrations of vitamin B-12 and MMA suggests that the activity (or the expression) of fetal methylmalonyl-CoA mutase may be enhanced by increasing maternal intake of vitamin B-12. The effect of vitamin B-12 supplementation during pregnancy on fetal MMA needs further investigation.

Concentrations of cystathionine were higher in the mothers in the current study than in our reference population of nonpregnant women or in our previous investigations of healthy individuals (10). Because our study included a normal group of pregnant women and their infants, elevated cystathionine is probably physiologic. Elevated cystathionine in addition to low PLP in the third trimester and in fetal blood suggest a slower rate of the transsulfuration pathway. The mean concentration of cystathionine was higher in fetal blood than in the mothers (Table 2). The mean concentration of cystathionine in cord blood from our subjects was comparable with the values in a recent study of Brazilian women (19). High concentrations of cystathionine have also been reported in newborns (aged ≈4 d) (17).

Cystathionase activity is absent in human fetal liver tissues (35) and is dependent on pre- and postnatal age (36, 37). Hepatic transsulfuration activity was reported to be reduced at birth, especially in premature infants (36, 37). Although all newborns in our study were full-term, we observed a significant correlation between gestational age and concentrations of cystathionine in cord serum. Cystathioninuria in premature infants has been shown to improve after vitamin B-6 treatment, which is known to enhance the activity of cystathionase (37). These data indicate that higher cystathionine at birth might be related to incomplete activities of the enzymes that mediate the transsulfuration pathway. Interestingly, markers of the transsulfuration pathway (PLP...
or cystathionine) were related to higher vitamin B-12 and vitamin B-6, respectively. The effect of maternal supplementation with vitamin B-12, vitamin B-6, or both during pregnancy on the newborn concentration of the metabolites should be investigated.

In summary, the results of our study show that umbilical cord concentrations of MMA and cystathionine can be predicted by maternal concentrations. Lower cord concentrations of MMA and cystathionine were related to higher vitamin B-12 and vitamin B-6, respectively. The effect of maternal supplementation with vitamin B-12, vitamin B-6, or both during pregnancy on the newborn concentration of the metabolites should be investigated.

We gratefully thank the staff members at the Department of Obstetrics and Gynecology at the University Hospital of Saarland for their cooperation. RO had the original idea for the study, participated in conceptualizing and designing the study, performed the biochemical and the statistical analyses, participated in the interpretation of the data, and wrote the manuscript. WM was responsible for recruiting the pregnant women for the study and participated in the study design, sample collection, and gathering of clinical data. MJ participated in collecting samples and gathering the clinical data. WS participated in recruiting the pregnant women for the study, WH supervised the study, participated in the design of the study and data interpretation, and provided input into the final draft of the manuscript. The authors had no personal or financial interests in any organization sponsoring this research and no conflicts of interest related to participation in this article.

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A follow-up study of nutrient intake, nutritional status, and growth in infants with cow milk allergy fed either a soy formula or an extensively hydrolyzed whey formula

Leena Seppo, Riitta Korpela, Bo Lönnerdal, Leena Metsäniitty, Kaisu Juntunen-Backman, Timo Klemola, Aila Paganus, and Timo Vanto

ABSTRACT

Background: Infants with cow milk allergy (CMA) are reported to have reduced growth and special nutritional needs.

Objective: The aim of the present study was to compare nutrient intake, nutritional status, and growth in infants with CMA who were fed either a soy formula or an extensively hydrolyzed whey formula.

Design: The study group comprised 168 double-blind challenge-proven infants with CMA. Eighty-four of the infants were fed a soy formula (mean starting age: 7.8 mo), and the other 84 infants were fed an extensively hydrolyzed whey formula (mean starting age: 7.5 mo).

Results: The length (SD score) of the infants was close to the mean Finnish reference growth by age 2 y in both groups. Weight-for-length measurements continued to reach the 50th percentile by age 4 y in both study groups. The mean nutrient intake followed the recommended intake in both groups, although most of the infants were supplemented with calcium and vitamin D. The observed serum transferrin receptor concentrations indicated a greater iron inadequacy in the tissue of infants in the soy formula group than in the hydrolyzed whey formula group ($P = 0.08$). However, there were no significant differences between the groups either in the percentages of abnormally low laboratory values (mean cell volume, hemoglobin, zinc, and ferritin) or in the percentages of high alkaline phosphatase activity, which indicates the comparable safety and effectiveness of the formulas studied.

Conclusions: Both nutritional status and growth were well within reference values in the 2 groups, and the selection of a formula can largely be made on the basis of infant tolerance to the formulas. *Am J Clin Nutr* 2005;82:140–5.

KEY WORDS Infant nutrition, cow milk allergy, extensively hydrolyzed whey formula, soy formula

INTRODUCTION

Allergy to cow milk protein occurs in 2–3% of infants (1–4). Almost half of these infants begin to tolerate cow milk by the age of 2 y (5, 6).

After weaning from breast milk, infants with cow milk allergy (CMA) are usually given either an extensively hydrolyzed formula or a soy formula. Soy formulas have a long history as alternative formulas for infants who are allergic to cow milk. Eight to 14% of infants with symptoms of immunoglobulin E (IgE)–associated CMA also react adversely to soy (7), but reports of anaphylaxis to soy are rare. Previously, we performed a randomized controlled study of infants with CMA in which 10% of the infants randomly assigned to a soy formula had adverse reactions from the formula compared with 2% of those randomly assigned to an extensively hydrolyzed formula (8). In some studies, growth was impaired in infants with CMA compared with healthy children (9–11), whereas in other studies growth reached the average rate after consumption of an elimination diet (12–14). The aim of the present study was to compare the effects of a soy formula or an extensively hydrolyzed whey formula on the nutritional status of infants until age 2 y and on their growth until age 4 y.

SUBJECTS AND METHODS

Subjects

The study involved 168 infants with CMA. The allergy was confirmed by a double-blind, placebo-controlled milk challenge, except in the case of 2 subjects who had a history of anaphylactic reaction to cow milk and who also had a positive skin-prick test and IgE antibodies to cow milk (15). Positive reactions that occurred within 2 h of the last challenge dose in the hospital were classified as immediate reactions; positive reactions that occurred later during the 5 d of challenge were classified as delayed reactions. At inclusion in the study, the symptoms that occurred most often in the infants because of CMA were atopic eczema

1 From the Foundation for Nutrition Research, Helsinki, Finland (LS and RK); Valio Ltd, Helsinki, Finland (LS, RK, and LM); the Institute of Biomedicine, Department of Pharmacology, University of Helsinki, Finland (RK); the Department of Nutrition, the University of California, Davis, CA (BL); the Skin and Allergy Hospital, Department of Allergology (KJ-B and TK) and the Hospital for Children and Adolescents (AP), Helsinki University Central Hospital, Helsinki, Finland; and the Turku University Hospital, Department of Pediatrics, Turku, Finland (TV).

2 Supported by grants from the Turku University Hospital, Turku, Finland, and the Social Insurance Institute and the University Hospital for Skin and Allergic Diseases, Helsinki, Finland. The extensively hydrolyzed whey formula (PeptidiTutteli) and the soy formula (SoijaTutteli) were provided by Valio Ltd, Helsinki, Finland.

3 Address reprint requests to L. Seppo, Foundation for Nutrition Research, PO Box 30, Helsinki FIN-00370, Finland. E-mail: leena.seppo@valio.fi. Received August 25, 2004. Accepted for publication March 21, 2005.
TABLE 1
Baseline characteristics of the subjects in the soy formula group (SFG) and the hydrolyzed whey formula group (HWFG)\(^{1}\)

<table>
<thead>
<tr>
<th></th>
<th>SFG (n = 84)</th>
<th>HWFG (n = 84)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>33</td>
<td>47</td>
</tr>
<tr>
<td>Female</td>
<td>51</td>
<td>37</td>
</tr>
<tr>
<td>Age (mo)</td>
<td>7.8 ± 2.1</td>
<td>7.5 ± 2.2</td>
</tr>
<tr>
<td>Immediate</td>
<td>47</td>
<td>55</td>
</tr>
<tr>
<td>Delayed</td>
<td>36</td>
<td>28</td>
</tr>
<tr>
<td>No challenge</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Skin-prick test (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–2 mm</td>
<td>44</td>
<td>49</td>
</tr>
<tr>
<td>≥3 mm</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>IgE antibodies to cow milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;0.35 kU/L</td>
<td>36</td>
<td>39</td>
</tr>
<tr>
<td>≥0.35 kU/L</td>
<td>38</td>
<td>34</td>
</tr>
</tbody>
</table>

\(^{1}\) In 2 subjects, cow milk allergy was confirmed on the basis of a history of anaphylactic reaction to cow milk, a positive result from a skin-prick test, and the presence of immunoglobulin E (IgE) antibodies to cow milk. There were no significant differences between groups (independent-samples t test for age and chi-square test for the other variables).

TABLE 2
Composition of study formulas\(^{1}\)

<table>
<thead>
<tr>
<th></th>
<th>Soy formula</th>
<th>Hydrolyzed whey formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ)</td>
<td>285</td>
<td>280</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>1.9</td>
<td>1.6</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>7.0</td>
<td>7.3</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>65</td>
<td>51</td>
</tr>
<tr>
<td>Phosphorus (mg)</td>
<td>42</td>
<td>30</td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>5.9</td>
<td>5.2</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>Copper (mg)</td>
<td>52</td>
<td>45</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>7.2</td>
<td>7.8</td>
</tr>
<tr>
<td>Riboflavin (μg)</td>
<td>78</td>
<td>130</td>
</tr>
</tbody>
</table>

\(^{1}\) Mean analyzed concentration per 100 mL prepared product.

(59%) and gastrointestinal symptoms (22%). When CMA was confirmed, the infants were allocated to groups that received either a soy formula (SoijaTutteli; Valio Ltd, Helsinki, Finland) or an extensively hydrolyzed whey formula (PeptidiTutteli; Valio Ltd) according to a computer-generated block randomization list; a block size of 4 was used. The participating hospital (of the 2 hospitals) and the age group (0–2, 3–5, 6–8, and 9–11 mo) were used as stratification variable factors in the randomization. The baseline characteristics of the infants are presented in Table 1.

The study protocol was approved by the Joint Commission on Ethics of Turku University and Turku University Central Hospital and by the Commission on Ethics of the Skin and Allergy Hospital, Helsinki University Central Hospital.

Study design

Twenty-one of 84 infants (25%) in the soy formula group (SFG) and 5 of 84 infants (6%) in the hydrolyzed whey formula group (HWFG) switched to another formula before the age of 2 y according to general health counseling in Finland. Calcium powder or tablets (250–500 mg) was recommended for all infants up to age 2 y according to general health counseling in Finland for infants who are allergic to cow milk products. Otherwise, the Finnish well-baby clinics’ guidelines for supplementary feeding were followed.

Both formulas complied with the nutrient recommendations of the European Union for infant formulas (16). The composition of the study formulas is presented in Table 2. In the extensively hydrolyzed whey formula, the size of all the peptides was <1600 Da. The phytate content of the soy-based formula was 4.1 μmol/100 g powder.

Calculation of nutrient intake

A 2-d dietary recall was completed by the infants’ parents or by daycare personnel when the infants were 1 and 2 y old. The NUTRICA program with a Finnish database (17), which was compiled from the manufacturer’s data and family recipes, was used for the calculation of energy and the contents of protein, fat, carbohydrate, calcium, riboflavin, iron, zinc, and vitamin E.

Laboratory analyses

Venous blood samples were obtained from the infants before the start of the study and at the ages of 1 and 2 y. The laboratory values of 6 infants who had received a formula for <1 mo by age 1 y were excluded from the analyses. The laboratory values for infants who discontinued using the study formula because of adverse reactions were included in the analyses at the age at which they were before the formula change. Serum alkaline phosphatase activity was determined by a kinetic method; hemoglobin concentrations, red blood cell indexes, and leukocyte concentrations were measured with the use of a Coulter Counter T-890 (Coulter Electronics, Tokyo, Japan); and serum calcium concentrations were measured with the use of a Hitachi 917 AutoAnalyzer (Hitachi Ltd, Tokyo, Japan). Serum ferritin concentrations were measured with a radioimmunoassay (Diagnostic Products, Los Angeles, CA), and serum transferrin receptor concentrations were measured with an enzyme-linked immunosorbent assay (Ramco, Houston, TX). After the dilution of blood samples with 10% nitric acid (1:5, by vol), serum zinc and...
copper concentrations were measured by atomic absorption spectrometry and with the use of National Institute of Standards and Technology (Gaithersburg, MD) standards.

Growth

The weight of the infants was measured with the use of electronic scales. The length of the infants who were up to age 2 y was measured by an experienced study nurse using a stadiometer while the infants were in a supine position. At the ages of 3 and 4 y, the standing height of each child was measured. The growth values of 6 infants who had received a formula for <1 mo by age 1 y were excluded from the analyses. Only growth values for those infants who did not change study formulas because of adverse reactions were included in the analyses. Length in SD score (SDS) and weight-for-length (% wt), which were expressed as the percentage deviation from the median weight for length and sex, were calculated with the use of the Finnish reference growth data (18). Length and weight were measured at the ages of 0, 6, 8, 10, 12, 18, 24, 36, and 48 mo.

Statistical analyses

For length (as SDS and in cm) and weight (percentage of weight-for-length and in kg), repeated laboratory measurements, and nutrient intake, the results are expressed as means ± SDs or means ± SEMs. Length as SDS and percentage of weight-for-length are the primary variables to indicate growth. Weight (in kg) and length (in cm) were considered secondary variables because the measurements of all the children were not taken at the exact predefined ages of 6, 8, 10, 12, 18, 24, 36, and 48 mo, and the actual length (in cm) and weight (in kg) may cause bias to the analysis. The differences in weight and length were not significant between the groups. For the length (SDS) and weight (percentage of weight-for-length) of infants aged 1–4 y, an analysis of variance (ANOVA) for repeated measures was performed as a primary analysis. The comparisons between the SFG and the HWFG are shown as mean differences with 95% CIs. The weight and length of infants aged <12 mo were not analyzed because consumption of the study formula began between the ages 2 and 11 mo.

Nutrient intake and laboratory results at ages 1 and 2 y were also analyzed with an ANOVA for repeated measures, and the corresponding baseline value was included as a covariate when appropriate. The differences between the groups (SFG compared with HWFG) are shown as means with 95% CIs. The association between laboratory measurements and nutrient intake was assessed with the use of Pearson’s correlation coefficients. The data were analyzed by using SPSS (version 12.0; SPSS Inc, Chicago, IL).

The decision to analyze the iron, zinc, and copper status was made post hoc, ie, not until the clinical study had been finished. At this time all the samples had been collected, and specific informed consent could not be obtained. The samples were analyzed without any patient identifiers at the University of California, Davis. Written informed consent was obtained from the infants’ parents for all the other parts of the present study.

RESULTS

At age 1 y, the mean (±SD) daily intake of the formula was 580 ± 194 mL in the SFG and 559 ± 267 mL in the HWFG. At age 2 y, 70% of the infants were still being fed the study formulas, and the mean (±SD) daily intake of formula was 420 ± 199 mL in the SFG and 369 ± 221 mL in the HWFG.

Nutrient intake

The infants in the SFG received significantly more percentage of energy from the study formula than did the infants in the HWFG (Table 3). Dietary intakes of zinc and vitamin E were significantly lower in the HWFG infants than in the SFG infants, and the intake of riboflavin was significantly higher in the HWFG than in the SFG.

Laboratory results

Laboratory results are presented in Table 4. The concentrations of transferrin receptors tended to indicate a greater tissue need for iron in the SFG than in the HWFG when analyzed by an ANOVA for repeated measures (P = 0.08).

### Table 3

Daily nutrient intakes of children with cow milk allergy in the soy formula group (SFG) and the hydrolyzed whey formula group (HWFG) at ages 1 and 2 y

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>SFG (n = 75–77)</th>
<th>HWFG (n = 69–71)</th>
<th>SFG (n = 70–73)</th>
<th>HWFG (n = 69–71)</th>
<th>Mean difference (95% CI)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ/kg)</td>
<td>387 ± 76²</td>
<td>384 ± 79</td>
<td>364 ± 87</td>
<td>372 ± 70</td>
<td>−3 (−25, 19)</td>
<td>0.77</td>
</tr>
<tr>
<td>Energy from the study formula (%)</td>
<td>45 ± 14</td>
<td>41 ± 19</td>
<td>22 ± 15</td>
<td>14 ± 15</td>
<td>6 (1, 11)</td>
<td>0.03</td>
</tr>
<tr>
<td>Protein (g/kg)</td>
<td>2.6 ± 0.6</td>
<td>2.6 ± 0.7</td>
<td>3.0 ± 0.9</td>
<td>3.1 ± 0.9</td>
<td>−0.1 (−0.3, 0.2)</td>
<td>0.55</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>33 ± 9</td>
<td>32 ± 10</td>
<td>40 ± 12</td>
<td>39 ± 10</td>
<td>1 (−2, 3)</td>
<td>0.58</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>125 ± 25</td>
<td>125 ± 26</td>
<td>139 ± 37</td>
<td>144 ± 28</td>
<td>−5 (−13, 3)</td>
<td>0.23</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>518 ± 166</td>
<td>449 ± 176</td>
<td>506 ± 262</td>
<td>464 ± 338</td>
<td>−49 (−18, 116)</td>
<td>0.15</td>
</tr>
<tr>
<td>Riboflavin (mg)</td>
<td>0.82 ± 0.22</td>
<td>0.98 ± 0.33</td>
<td>1.03 ± 0.43</td>
<td>1.11 ± 0.55</td>
<td>−0.14 (−0.26, −0.02)</td>
<td>0.02</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>9.7 ± 2.4</td>
<td>8.8 ± 2.2</td>
<td>9.1 ± 3.1</td>
<td>8.6 ± 2.3</td>
<td>0.6 (−0.05, 1.26)</td>
<td>0.07</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>7.9 ± 2.7</td>
<td>5.9 ± 1.5</td>
<td>8.0 ± 2.6</td>
<td>6.7 ± 1.5</td>
<td>1.7 (1.10, 2.21)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>9.9 ± 2.7</td>
<td>8.0 ± 2.7</td>
<td>8.2 ± 3.8</td>
<td>6.9 ± 2.6</td>
<td>1.6 (0.8, 2.4)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

¹ ± SD (all such values).
² Repeated-measures ANOVA. The interaction between group and time was not significant for all variables.
³ Values do not include intake from supplements.
The percentages of low ferritin (<12 μg/L), hemoglobin (<110 g/L), mean cell volume (<75 fl), and zinc (<0.70 mg/L) concentrations and the percentage of high serum alkaline phosphatase (>1000 U/L) concentrations in the 2 groups are presented in Table 4. There were no significant differences between the groups, nor was there any relation between the concentrations of zinc and ferritin (κ coefficient = 0.02, P = 0.89). No correlation between dietary intake and the results of the biochemical analysis was found.

**Growth**

Length (SDS) and percentage of weight-for-length at 0, 6, 10, 12, 18, 24, 36, and 48 mo of age are shown in Figure 1. The growth data (length in SDS and in cm, and weight as a percentage of weight-for-length and in kg) were not significantly different between the groups at baseline. The growth data from 1 to 4 y were analyzed with an ANOVA for repeated measures. In all of the analyses, the interactions (group × time) were not significant (P = 0.723 for SDS, P = 0.260 for cm, P = 0.664 for percentage of weight-for-length, and P = 0.627 for kg).

The mean length as SDS during that period was 0.10 in the SFG and −0.03 in the HWFG. The mean length in cm was 89.6 in the SFG and 89.4 in the HWFG. The mean difference between the groups was 0.13 SDS (95% CI: −0.18, 0.44; P = 0.392) or 0.2 cm (95% CI: −0.9, 1.4; P = 0.682).

The mean weight as a percentage of weight-for-length during the period from 1 to 4 y was −1.8% in the SFG and −3.2% in the HWFG. The mean weight was 13.2 kg in the SFG and 13.0 kg in the HWFG. The mean difference between the groups was 1.4% (95% CI: −0.7, 3.5; P = 0.191) or 0.3 kg (95% CI: −0.2, 0.7; P = 0.301).

Forty infants in the SFG and 48 infants in the HWFG had immediate, often IgE–mediated, reactions to cow milk; 25 infants in the SFG and 27 infants in the HWFG had delayed, often non-IgE–mediated, reactions. There were no significant differences in weight or length between the infants with immediate reactions to cow milk and those with delayed reactions in the SFG or in the HWFG.

A percentage of weight-for-length value of <−15% was found in 4 infants (6.1%) in the SFG and 5 infants (6.6%) in the HWFG who were between the ages of 1 and 4 y. The age when the infants began to ingest the study formula did not correlate with growth by ages 1 or 2 y.

**DISCUSSION**

As far as we know, no previous prospective randomized studies have compared the effects of the 2 types of formula used in the present study on the nutritional status and growth of infants. Nutritional status and growth were well within the reference values in the 2 groups, and the selection of a formula can largely be based on the infant’s tolerance to the formulas. Adverse reactions to the soy formula occurred more often than did adverse reactions to the hydrolyzed formula (10% compared with 2%), but these reactions were seldom severe or IgE–mediated (8). On the other hand, soy formulas are cheaper and probably more palatable, which was indicated by the slightly higher intake of the soy formula than of the extensively hydrolyzed formula and by the good catch-up growth in the SFG.

**TABLE 4**

<table>
<thead>
<tr>
<th>Laboratory results for infants with cow milk allergy in the soy formula group (SFG) and the hydrolyzed whey formula group (HWFG) at baseline and at ages 1 and 2 y</th>
<th>Baseline</th>
<th>1 y</th>
<th>2 y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SFG (n = 53)</td>
<td>HWFG (n = 63)</td>
<td>SFG (n = 51)</td>
</tr>
<tr>
<td>Transferrin receptor (mg/L)</td>
<td>6.4 ± 2.1³</td>
<td>5.8 ± 1.8³</td>
<td>6.6 ± 1.4³</td>
</tr>
<tr>
<td>Ferritin (µg/L)²</td>
<td>61 ± 50⁴</td>
<td>88 ± 95⁴</td>
<td>30 ± 16³</td>
</tr>
<tr>
<td>&lt;12 µg/L (%)</td>
<td>11</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>Zinc (mg/L)</td>
<td>0.71 ± 0.13³</td>
<td>0.69 ± 0.14³</td>
<td>0.67 ± 0.09³</td>
</tr>
<tr>
<td>&lt;0.70 mg/L (%)</td>
<td>15</td>
<td>11</td>
<td>21</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>115 ± 8</td>
<td>117 ± 8</td>
<td>117 ± 7</td>
</tr>
<tr>
<td>&lt;110 g/L (%)</td>
<td>21</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>78 ± 4</td>
<td>80 ± 4</td>
<td>79 ± 4</td>
</tr>
<tr>
<td>&lt;75 fl (%)</td>
<td>17</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>S-AFOS (U/L)</td>
<td>643 ± 284</td>
<td>834 ± 1297</td>
<td>635 ± 198</td>
</tr>
<tr>
<td>&gt;1000 U/L (%)</td>
<td>4</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Calcium (mmol/L)</td>
<td>2.61 ± 0.08</td>
<td>2.62 ± 0.07</td>
<td>2.56 ± 0.08</td>
</tr>
<tr>
<td>Copper (µg/L)</td>
<td>0.9 ± 0.2³</td>
<td>1.0 ± 0.2³</td>
<td>1.1 ± 0.2³</td>
</tr>
</tbody>
</table>

¹ Data for infants who did not change study formulas because they had no adverse reactions and those who did but who received the study formula for ≥1 mo by the age of 1 y are included. Hb, hemoglobin; MCV, mean cell volume; S-AFOS, serum alkaline phosphatase. The interaction between group and time was not significant for all variables.

² Repeated-measures ANOVA, with the baseline value included as a covariate.

³ n = 47.

⁴ n = 56.

⁵ n = 42.

⁶ n = 52.

⁷ n = 36.

⁸ Significantly different from baseline SFG, P < 0.05.
It has been suggested that infants with food allergies need more nutrients than do healthy infants because of their impaired ability to utilize nutrients, which may be caused by allergy-induced bowel inflammation (19). In the present study, the negative effects on growth in infants with CMA were seen before an appropriate diet was substituted; however, catch-up growth was good in the infants after the substitution of cow milk with either the soy formula or the hydrolyzed whey formula. In our study, the energy intake of the infants was 144 SEPPO ET AL

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In conclusion, the length growth of infants with CMA reached the mean Finnish reference value by age 2 y in both groups. Weight-for-length measurements in the infants remained below the 50th percentile by age 2 y. However, the weight-for-length measurements in the infants continued to reach the 50th percentile by age 4 y in both study groups. The mean intake of nutrients and nutritional status followed recommendations; however, individual dietary counseling was needed in many cases. Even with such counseling, it appears that nutritional status and growth did not differ between the groups, and the selection of a formula can therefore largely be made on the basis of tolerability and, to some extent, on cost.

We thank Antti Koivikko, Pirkko Syvänén, and Erkka Valovirta for performing the clinical examinations and for supervising the food challenges. We are grateful to Marja Marja-aho and Janette Rääkkönen for their skillful handling of the food challenges, Janette Rääkkönen for performing the skin-prick tests, Tuija Poussa for her expert assistance with the statistical analyses, Anneli Pere for useful comments on the manuscript, and Mimi Ponsonby for correcting the manuscript.

TV, KJ-B, TK, and RK were the principal investigators of the study. BL analyzed serum zinc, copper, ferritin, and transferrin receptor concentrations. LS, RK, and TV prepared the manuscript. LS counted the intake of nutrients. LM and AP were the infant nutrition specialists for this study. KJ-B, TK, and BL contributed to the preparation of the manuscript. None of the authors reported any conflicts of interest.

REFERENCES

Postprandial response to a physiologic caloric load in HIV-positive patients receiving protease inhibitor–based or nonnucleoside reverse transcriptase inhibitor–based antiretroviral therapy1–3


ABSTRACT
Background: Features of the dyslipidemic pattern reported with the use of antiretroviral therapy predict enhanced postprandial lipemia, which is an emerging cardiovascular disease risk factor.

Objective: We evaluated the postprandial response to a physiologic, meal-based challenge in HIV-positive subjects without hyperlipidemia.

Design: We measured hourly lipid, lipoprotein, glucose, and insulin concentrations during a 13-h period in 25 nonwhite patients (13 women, 12 men): 13 receiving a protease inhibitor (PI)-based regimen (6 nelfinavir and 7 indinavir) and 12 receiving a nonnucleoside reverse transcriptase inhibitor (NNRTI)-based regimen (6 efavirenz and 6 nevirapine).

Results: Mean fasting HDL-cholesterol concentrations were lower in HIV patients than in healthy subjects without HIV infection matched for age, sex, and ethnicity (z score: −0.81 ± 0.9; P = 0.0001). Fasting triacylglycerol concentrations were not significantly different between HIV-infected patients and healthy subjects but were higher in PI-treated than in NNRTI-treated patients [median (interquartile range): 144 (110–191) and 89 (62–135) mg/dL; P = 0.007]. Average daylong triacylglycerol concentrations, but not incremental concentrations, were higher in the PI group than in the NNRTI group [205% (185–248%) and 125% (78–191%); P < 0.05]. For all HIV-positive patients, the fractional triacylglycerol increase was lower after breakfast than after lunch (20 ± 18% and 42 ± 40%, respectively; P < 0.04). Insulin concentrations were higher in PI-treated than in NNRTI-treated patients [22.6 (13.1–29.8) and 11.8 (7.1–19.1) μU/mL; P = 0.01] and increased in both groups in response to each meal, whereas glucose concentrations increased only after breakfast.

Conclusions: Despite baseline differences, incremental triacylglycerol and insulin responses to a physiologic caloric load among HIV-positive patients were not significantly affected by differences in the type of antiretroviral therapy. Am J Clin Nutr 2005;82:146–54.

KEY WORDS HIV, protease inhibitors, nonnucleoside reverse transcriptase inhibitor, NNRTI, antiretroviral treatment, ART, postprandial lipemia, insulin resistance, African Americans

INTRODUCTION
The introduction of potent antiretroviral therapy (ART) represents a major breakthrough in HIV treatment and has resulted in considerable improvements in both morbidity and mortality (1–6). However, it has become evident that the use of ART is associated with significant metabolic side effects, such as body fat redistribution, hyperlipidemia, and insulin resistance (7–11). Although variable lipid and lipoprotein changes have been reported in patients undergoing HIV ART (12–21), the most common lipoprotein pattern reported to date is characterized by decreased HDL-cholesterol concentrations, increased triacylglycerol concentrations, and a modest increase in LDL-cholesterol concentrations (9–11). This lipid pattern has similarities with the lipoprotein phenotype observed in patients with the metabolic syndrome (22, 23). In addition, the metabolic syndrome is characterized by the presence of insulin resistance, a pattern also frequently found during ART (9–11, 14). Studies have shown accelerated atherosclerosis and cardiovascular disease during ART of HIV-infected patients, which agrees with the notion of the metabolic syndrome as an atherogenic phenotype (22–27).

In addition to fasting lipid concentrations, postprandial lipemia has been indicated as an emerging cardiovascular disease risk factor (22, 28). Although fasting lipid concentrations determine hypolipidemic treatment decisions, postprandial lipemia is used primarily as an indicator of risk rather than as a guide to therapy. So far, although there is a wealth of information on fasting lipid concentrations, relatively few studies have focused on postprandial lipemia (28–30), and this condition remains...
virtually unexplored in HIV ART. Interestingly, several components of the lipid profile that are associated with both the metabolic syndrome and the use of ART in HIV-infected patients, such as decreased HDL cholesterol and increased triacylglycerol concentrations, have been suggested as predictors of the postprandial lipid response (28–32). Furthermore, most of the postprandial studies undertaken to date have used conditions involving a metabolic stress, such as a high-fat load (28–30). Although the postprandial state following physiologic meal intakes represents the norm during daytime, investigations addressing the response to physiologic caloric loads are scarce.

To reflect a physiologic state, we evaluated the response to defined, standardized meals representing a natural food consumption pattern in a cohort of normolipidemic African American and Hispanic HIV-infected patients receiving ART. Because differences in fasting lipid and lipoprotein concentrations have been noted in response to different ART regimens, such as protease inhibitor (PI)-based compared with nonnucleoside reverse transcriptase inhibitor (NNRTI)-based regimens (33, 34), we hypothesized that such baseline differences in lipid concentrations would translate into a different postprandial response in patients receiving a PI-based or an NNRTI-based antiretroviral regimen.

SUBJECTS AND METHODS

Patients

HIV-positive African American and Hispanic patients were recruited from outpatient HIV clinics at Harlem Hospital Center in New York. Eligibility for enrollment in the study was based on the presence of documented HIV infection, ongoing stable antiretroviral regimen for >6 mo, and the absence of hyperlipidemia. Patients were excluded if they had a history of diabetes mellitus, had been receiving ART for <6 mo, were pregnant, had fasting concentrations <11 mg/dL, had a history of cirrhosis of the liver, had any opportunistic infections that required treatment in the past 30 d, were pregnant, had fasting cholesterol or triacylglycerol concentrations >200 mg/dL, were receiving hypolipidemic therapy, had Cushing syndrome or untreated hypogonadism, or were being treated with growth hormone, anabolic steroids (including dehydroepiandrosterone), ketoconazole, or corticosteroids. The study was approved by the Institutional Review Boards at Columbia University, Harlem Hospital Center, St Luke’s–Roosevelt Medical Center, VA Northern California Health Care System, and University of California, Davis, and informed consent was obtained from all participants.

Overall, 25 patients were recruited for the study: 12 men and 13 women. Twenty-three patients were African American and 2 were Hispanic; 13 patients were undergoing PI-based ART (6 on nelfinavir and 7 on indinavir), and 12 patients were undergoing NNRTI-based ART (6 each on nevirapine and efavirenz). The type of antiretroviral regimen was defined as PI-based for patients taking 2 or more nucleoside reverse transcriptase inhibitors (NRTIs) in combination with at least 1 PI or a regimen containing ritonavir and saquinavir in combination with 1 NRTI and no NNRTIs. NNRTI-based ART regimens were combinations of ≥2 NRTIs in combination with ≥1 NNRTI (lamivudine, stavudine, didanosine, or zidovudine/lamivudine with efavirenz or nevirapine) (19). Adherence to therapy was gauged by history and follow-up with the primary care provider. The CD4 count range was 250−1240 (± 654); viral load was undetectable in 15 patients and <2900 in 10 patients. All patients had been receiving ART for >6 mo.

Study design

The subjects were admitted to the Columbia University General Clinical Research Center (GCRC) in the evening. They continued their respective standard ART regimen throughout the study. After admittance, the patients fasted until the morning breakfast at 0900 the following day. At 0700, an indwelling catheter was placed and kept patent by a saline infusion. The first blood draw was carried out at 0800, which was followed by hourly blood samples until 2000. At meal times, the blood draw was obtained before serving the meal. Meals were served at the following times: breakfast at 0900, lunch at 1200, and dinner at 1700. All meals were prepared by the GCRC Bionutrition Unit as described below. After the blood sample taken at 2000, the catheter was removed and the patients discharged. Because the first meal was given after the 1-h blood sample, we defined the baseline concentrations as the average of the 0-h and 1-h time points.

Comparison with the DELTA Study

Results from HIV-infected patients enrolled in the present study were compared with a previously conducted diet study in HIV-negative subjects, the DELTA (Dietary Effects on Lipoproteins and Thrombogenic Activities) Study (35, 36). As part of that study, 2 separate feeding protocols were carried out. In the second DELTA study protocol, DELTA 2, subjects aged 21–68 y of different ethnicity, including African American and Hispanic, and having characteristics of the metabolic syndrome (low HDL-cholesterol, high triacylglycerol, or high insulin concentrations) were recruited to participate in a multicenter, randomized, double-blind study with a 3-period crossover design. None of the participants took any medications known to affect plasma lipids or hemostatic factors. Eligibility was based on meeting one or more of the following requirements: 1) HDL cholesterol ≥ 30th percentile, 2) triacylglycerols ≥ 90th percentile, or 3) insulin ≥ 70th percentile, all adjusted for age, sex, and ethnicity. In the DELTA 2 study, the subjects were fed 3 different diets for 7–8 wk [average American diet, American Heart Association (AHA) Step I diet, and a high-monounsaturated-fat diet]. As part of the DELTA 2 protocol, blood samples were drawn during fasting conditions, before lunch, and before dinner (fasting, 4 h, and 8 h) in 68 participants (43 men and 25 women) with a mean age of 34.8 y (range: 22–61 y) and body mass index (BMI; in kg/m²) of 27.6 ± 4.4 (± SD), and insulin, glucose, and triacylglycerol concentrations at those time points were assessed. The values at these time points during the AHA Step I diet were compared with the concentrations from HIV-positive subjects obtained in the present study at the corresponding time points. The DELTA Study was designed for weight maintenance, and menus were designed with 150-kcal increments from 1500 to 3000 kcal that allowed for interindividual variations in total energy intake. The menu choice was the same as for the HIV-positive subjects and the macronutrient composition was identical. The calorie distribution for a 2000-kcal diet was equalized over the day with 650 kcal for each meal.
Diets

The diet composition and the distribution of calories over the meals used in the present study are summarized in Table 1. The Bionutrition Unit in the Columbia University GCRC developed menus for 3 different caloric levels (2000, 2200, and 2500 kcal) with the use of standardized recipes. For each meal, the food was weighed before and after it was consumed, and the amounts of calories consumed were calculated. As seen in Table 1, about 90% of the calories provided were consumed, which resulted in a mean caloric intake of 1957 kcal/d. The caloric distribution was designed to provide 25–28% of total energy for breakfast, 35% for lunch, and 37–40% for dinner. The mean relative distribution of calories consumed during the day was 23.1% (breakfast), 39.7% (lunch), and 37.2% (dinner) for the PI group, and 27.3%, 35.8%, and 37.5%, respectively, for the NNRTI group.

The distribution of caloric content was based on the National Cholesterol Education Program (NCEP) Step I recommendations, with 55% of calories from carbohydrates, 15% from protein, and 30% from fat (37). The diets provided an average of 24 g fructose and 18 g fiber per day. The energy requirement of each individual participating in the study was calculated by using the Harris-Benedict equation for basal energy expenditure with an activity factor of 1.3 (38). The food intake of each subject and the menus provided (Table 2) were calculated by using the University of Minnesota (Minneapolis, MN) NUTRITION DATA SYSTEMS software program (version 4.02/30).

Laboratory analyses

Immediately after each blood draw, plasma and serum were separated by centrifugation at 30 000 × g for 20 min at 4 °C. The plasma and serum samples were aliquotted and immediately transferred to a −80 °C freezer where they were stored until analyzed. Plasma total, LDL-, and HDL-cholesterol; triacylglycerol; and glucose concentrations were measured by using standard enzymatic techniques as previously described (39). The CVs for these assays were 3–5%. The laboratory participates in the lipid and lipoprotein standardization program of the Centers for Disease Control and Prevention. Serum concentrations of apolipoproteins (apo) A-I and B were determined by immunonephelometric procedures on a Beckman Array 360 nephelometer with commercially available reagents (Beckman, Brea, CA) (39). The interassay CVs for these measurements were 3–5%. Plasma insulin concentrations were measured by using commercially available reagents without cross-reactivity with proinsulin concentrations (Linco Research, St Charles, MO). The interassay CV for this measurement was 6–8% (36).

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Diet composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy provided (kcal/d)</strong></td>
<td><strong>Value</strong></td>
</tr>
<tr>
<td>Total</td>
<td>2189</td>
</tr>
<tr>
<td>Breakfast</td>
<td>567</td>
</tr>
<tr>
<td>Lunch</td>
<td>788</td>
</tr>
<tr>
<td>Dinner</td>
<td>836</td>
</tr>
<tr>
<td><strong>Energy consumed (kcal/d)</strong></td>
<td><strong>Value</strong></td>
</tr>
<tr>
<td>Total</td>
<td>1957</td>
</tr>
<tr>
<td>Breakfast</td>
<td>488</td>
</tr>
<tr>
<td>Lunch</td>
<td>737</td>
</tr>
<tr>
<td>Dinner</td>
<td>733</td>
</tr>
<tr>
<td><strong>Calories consumed (%)</strong></td>
<td><strong>Value</strong></td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
</tr>
<tr>
<td>Breakfast</td>
<td>88</td>
</tr>
<tr>
<td>Lunch</td>
<td>94</td>
</tr>
<tr>
<td>Dinner</td>
<td>88</td>
</tr>
<tr>
<td><strong>Energy distribution</strong></td>
<td><strong>Value</strong></td>
</tr>
<tr>
<td>Carbohydrates (% of energy)</td>
<td>56</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>15</td>
</tr>
<tr>
<td>Fat (% of energy)</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td>268</td>
</tr>
<tr>
<td>Breakfast</td>
<td>85</td>
</tr>
<tr>
<td>Lunch</td>
<td>98</td>
</tr>
<tr>
<td>Dinner</td>
<td>93</td>
</tr>
<tr>
<td>Fructose (g)</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
</tr>
<tr>
<td>Breakfast</td>
<td>13</td>
</tr>
<tr>
<td>Lunch</td>
<td>5</td>
</tr>
<tr>
<td>Dinner</td>
<td>7</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
</tr>
<tr>
<td>Breakfast</td>
<td>5</td>
</tr>
<tr>
<td>Lunch</td>
<td>7</td>
</tr>
<tr>
<td>Dinner</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Dietary menu components</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breakfast</strong></td>
<td><strong>Value</strong></td>
</tr>
<tr>
<td>Banana bread with jelly</td>
<td></td>
</tr>
<tr>
<td>Skim milk</td>
<td></td>
</tr>
<tr>
<td>Decaffeinated coffee</td>
<td></td>
</tr>
<tr>
<td><strong>Lunch</strong></td>
<td><strong>Value</strong></td>
</tr>
<tr>
<td>Salad with dressing</td>
<td></td>
</tr>
<tr>
<td>Minestrone soup</td>
<td></td>
</tr>
<tr>
<td>Crackers</td>
<td></td>
</tr>
<tr>
<td>Turkey sandwich with spread</td>
<td></td>
</tr>
<tr>
<td>Apple juice</td>
<td></td>
</tr>
<tr>
<td>Angel food cake</td>
<td></td>
</tr>
<tr>
<td><strong>Dinner</strong></td>
<td><strong>Value</strong></td>
</tr>
<tr>
<td>Menu 1</td>
<td></td>
</tr>
<tr>
<td>Macaroni and cheese</td>
<td></td>
</tr>
<tr>
<td>Green beans</td>
<td></td>
</tr>
<tr>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td>Fruit ice</td>
<td></td>
</tr>
<tr>
<td>Menu 2</td>
<td></td>
</tr>
<tr>
<td>Breaded chicken breast with rice</td>
<td></td>
</tr>
<tr>
<td>Green beans</td>
<td></td>
</tr>
<tr>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td>Fruit ice</td>
<td></td>
</tr>
<tr>
<td>Applesauce tofu muffin</td>
<td></td>
</tr>
</tbody>
</table>
RESULTS

To explore whether differences in baseline lipoprotein, glucose, and insulin concentrations predicted the postprandial triacylglycerol response in normolipidemic HIV-positive patients, we analyzed these concentrations in relation to data for an HIV-negative population and then evaluated the relation between baseline concentrations and the triacylglycerol excursion level during the day in the HIV-positive subjects. Initially, we compared the fasting, baseline lipid, glucose, and insulin concentrations of the HIV groups with those of a healthy population. We calculated $z$ scores for triacylglycerols and HDL cholesterol with adjustment for age, sex, BMI, and race/ethnicity by using data from NHANES. As shown in Figure 1, the $z$ score for HDL cholesterol was significantly lower for the HIV subjects as a group ($P = 0.0001$), whereas no significant difference in $z$ score for triacylglycerols was found for the HIV-positive group.

Comparisons of baseline lipid values with those for the healthy population ($z$ score calculations) were also done separately for the PI and NNRTI groups. As shown in Figure 1, although the PI-treated group had a numerically lower $z$ score for HDL cholesterol than did the NNRTI group, the difference between the 2 groups was not significant. For triacylglycerols, there was a significant difference in $z$ scores between PI-treated patients and the NNRTI-treated patients ($P = 0.016$).

We then evaluated whether baseline and postprandial variables differed between PI-treated and NNRTI-treated HIV-positive patients. The clinical characteristics and fasting baseline lipid concentrations of the 2 groups of subjects are given in Table 3 and are compared with values for HIV-negative subjects with features of the metabolic syndrome from the DELTA Study. As shown in the table, PI-treated patients had a significantly higher mean BMI and fasting insulin and triacylglycerol concentrations than did the NNRTI-treated group. In contrast, total and LDL-cholesterol concentrations and glucose concentrations did not differ significantly at baseline between the 2 groups. Fasting apo A-I and HDL-cholesterol concentrations were higher in the

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**TABLE 3**

Clinical characteristics of the patients and control subjects

<table>
<thead>
<tr>
<th>HIV-positive patients</th>
<th>NNRTI-based ART</th>
<th>DELTA2 control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 5 M, 5 F)</td>
<td>(n = 7 M, 5 F)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>42.5 ± 8.8$^2$</td>
<td>42.7 ± 9.8$^2$</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>29.7 ± 5.9$^3$</td>
<td>25.8 ± 2.8</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>178.4 ± 37</td>
<td>183.2 ± 33</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>109.5 ± 29</td>
<td>117.0 ± 27</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>37.3 ± 14$^4$</td>
<td>46.0 ± 16$^4$</td>
</tr>
<tr>
<td>Triacylglycerols (mg/dL)</td>
<td>144 (110–191)$^5,6$</td>
<td>89 (62–135)$^3$</td>
</tr>
<tr>
<td>Apo A-I (mg/dL)</td>
<td>103.9 ± 20$^5$</td>
<td>118.4 ± 17</td>
</tr>
<tr>
<td>Apo B (mg/dL)</td>
<td>83.2 ± 17$^2$</td>
<td>71.2 ± 23$^2$</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>107 ± 21$^2$</td>
<td>97.6 ± 9</td>
</tr>
<tr>
<td>Insulin (µU/mL)</td>
<td>22.6 (13.1–29.8)$^2,5$</td>
<td>11.9 (7.1–19.1)</td>
</tr>
</tbody>
</table>

---

$^1$ All values are $\bar{x}$ ± SD unless noted otherwise. PI, protease inhibitor; ART, antiretroviral therapy; NNRTI, nonnucleoside reverse transcriptase inhibitor; DELTA2, Dietary Effects on Lipoproteins and Thrombogenic Activities Study (protocol 2); Apo, apolipoprotein. The statistical tests used were ANOVA for all continuous variables and the chi-square test for sex.

$^2,5$ Significantly different from the DELTA2 group: $^2P < 0.01$, $^5P < 0.05$.

$^3,5$ Significantly different from the NNRTI group: $^3P < 0.05$, $^5P < 0.01$.

$^6$ Median; interquartile range in parentheses (all such values).
NNRTI group. The mean BMI of 25.8 for the NNRTI group was at the borderline between values defining normal weight and overweight. In contrast, the PI patients had a mean BMI of 29.7, which is at the borderline between values defining overweight and obesity. The PI-treated group had metabolic laboratory values reminiscent of the metabolic syndrome, with triacylglycerol and glucose concentrations approaching the upper limit of the normal distribution and an HDL-cholesterol concentration below the level associated with cardiovascular disease risk. The PI-treated group had metabolic laboratory values for the triacylglycerol AUC over the day, whereas HDL-cholesterol concentrations correlated negatively. When the incremental AUC was dichotomized for the postbreakfast (1–4 h) and postlunch (4–9 h) periods, there was no significant association between any of the baseline lipid variables and the postbreakfast response, whereas all lipid variables except for apo A-I were significantly associated with the postlunch incremental AUC response. Baseline glucose and insulin concentrations were not significantly associated with the postprandial triacylglycerol response.

We then compared the difference in triacylglycerol concentrations at baseline and 4 and 8 h postprandially for HIV-positive patients and HIV-negative subjects with features of the metabolic syndrome as described in the Subjects and Methods. Overall, the triacylglycerol increase over 8 h was not significantly different for the 2 groups (Figure 2). However, the distribution of the increase over the day differed substantially. Compared with HIV-negative subjects, HIV-positive subjects had a lower fractional triacylglycerol increase after breakfast (20 ± 18% compared with 70 ± 42%, respectively; \(P < 0.0001\)) but a higher fractional triacylglycerol increase after lunch (42 ± 40% compared with −9 ± 27%, respectively; \(P < 0.0001\)). These results suggest a later triacylglycerol response in the ART-treated HIV-positive subjects than in the HIV-negative subjects. To assess possible effects of age, we performed a subgroup analysis by only including subjects from both studies within an overlapping age range (30–60 y); the mean ages of the subgroups were 39 ± 7 y (\(n = 42\)) for the DELTA subjects and 41 ± 6 y (\(n = 23\)) for the HIV-positive patients. The \(P\) values for the triacylglycerol AUC for the time periods 0–4 and 0–8 h remained <0.0001, as they were for the full groups.

The HIV-positive subjects had significantly higher insulin concentrations at baseline than did the HIV-negative subjects with features of the metabolic syndrome [median (interquartile range): 21.8 (8.6–28.7) and 10.4 (7.6–16.4) \(\mu\)U/mL; \(P = 0.0009\)], and the difference compared with the HIV-negative subjects remained at 4 and 8 h \((P = 0.009\) and \(P = 0.0008\), respectively; data not shown). No significant differences in glucose concentrations were noted by HIV status. Notably, within the HIV group, the fractional triacylglycerol increase differed significantly after breakfast and after lunch (20 ± 18% and 42 ± 40%; \(P < 0.04\)).

The daylong results for triacylglycerol, insulin, and glucose for the PI and NNRTI groups separately are presented in Figure 3. Although baseline concentrations differed, as described above, the excursion pattern for triacylglycerols was similar for the PI- and NNRTI-treated groups (Figure 3A). Thus, there was virtually no increase in triacylglycerol concentrations after the breakfast meal for either group, whereas triacylglycerol concentrations started to increase after the lunch meal, with a broad peak 3 h after lunch. Thereafter, triacylglycerol concentrations decreased and this declining pattern continued through the postdinner measurements. Although the PI group had higher triacylglycerol concentrations than did the NNRTI group, and a significantly higher AUC, the incremental AUC for triacylglycerols for the 2 groups did not differ significantly (Table 5). For both groups, the incremental AUC for the 4–8-h period was significantly higher than the corresponding AUC for the 0–4-h period (Table 5).

As shown in Table 3, fasting insulin concentrations were significantly higher among PI subjects than in the NNRTI group.
(median concentrations: 22.6 and 11.8 μU/mL; P = 0.01), and insulin concentrations remained higher throughout the day for the PI group, with a trend for a higher AUC (61.7 and 37.7 μU/mL; P = 0.07; Table 6). However, the incremental AUC for insulin did not differ significantly between the PI and NNRTI groups. In both groups, insulin concentrations fluctuated during

FIGURE 3. Mean (±SD) hourly concentrations of triacylglycerols (TG), insulin, and glucose in patients receiving a protease inhibitor–based antiretroviral therapy regimen (n = 13; ○) and in patients receiving a nonnucleoside reverse transcriptase inhibitor–based antiretroviral therapy regimen (n = 12; ■). Triacylglycerol and insulin concentrations were transformed by using logarithm and square root (Sqrt), respectively. Meals were provided after 1 h (breakfast), 4 h (lunch), and 9 h (dinner).

| TABLE 5 | Postprandial triacylglycerol excursions
<table>
<thead>
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<tbody>
<tr>
<td></td>
<td>AUC</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Triacylglycerols, 1–4 h</td>
<td>479 (391–672)</td>
</tr>
<tr>
<td>Triacylglycerols, 4–9 h</td>
<td>1187 (1030–1371)</td>
</tr>
</tbody>
</table>

* n = 13 for the PI group and n = 12 for the NNRTI group. AUC, area under the curve; PI, protease inhibitor; ART, antiretroviral therapy; NNRTI, nonnucleoside reverse transcriptase inhibitor.

1 Median; interquartile range in parentheses (all such values).

2 Significantly different from PI group (t test): 3P < 0.01, 5P < 0.05.

3 x ± SD (all such values).
The HIV-positive subjects in the present study therefore represents a closer image of the daily occurring postprandial state. Although there is a growing interest in evaluating daylong lipid measurements in the assessment of postprandial lipemia (40, 41), the overall experience from such studies is limited. On the basis of the results of traditional, high-fat load studies, it is well established that HDL-cholesterol and triacylglycerol concentrations are important predictors of the postprandial response (28–30). Both of these lipid fractions are variables used to define the metabolic syndrome, and persons with this syndrome are expected to have an increased potential for postprandial lipemia (22, 23). The HIV-positive subjects receiving ART who were evaluated in the present study had significantly lower HDL-cholesterol concentrations but similar triacylglycerol concentrations under fasting conditions as did control subjects matched for age, sex, BMI, and race/ethnicity. It is in this context of interest to note that the combination of low HDL-cholesterol and high triacylglycerol concentrations was reported in HIV-infected subjects before widespread use of ART (42). Furthermore, although mean fasting glucose concentrations were <110 mg/dL, below the cutoff included in the definition of the metabolic syndrome (23), insulin concentrations were high, which is compatible with some degree of insulin resistance. Thus, although frank hyperlipidemia was an exclusion criterion in the present study, the HIV subjects assessed had several of the characteristics associated with the metabolic syndrome.

To assess predictors of the postprandial triacylglycerol response in the HIV-positive patients under physiologic conditions, we tested for correlations between baseline lipid, apolipoprotein, glucose, and insulin concentrations and the incremental triacylglycerol AUC. Several lipid indexes, including triacylglycerol, HDL-cholesterol, LDL-cholesterol, and apo B concentrations, showed a significant correlation with the incremental triacylglycerol response postlunch. This response was not related to baseline glucose or insulin concentrations. Notably, several of the lipid indexes characterizing the dyslipidemia seen in HIV-infected patients receiving ART were among these predictors, which suggests that the degree of dyslipidemia observed in patients with HIV who are receiving ART may influence postprandial lipemia during physiologic conditions. Interestingly, the response was predicted by LDL-cholesterol or apo B concentrations, which under more pronounced stress-test conditions have not uniformly predicted postprandial lipemia (28, 31).

The HIV-positive subjects had a temporal postprandial triacylglycerol pattern in which the greatest increase occurred late in the day, with a well-defined peak shortly after each meal (Figure 3B). Glucose concentrations peaked after breakfast for both groups, being higher among the PI subjects, whereas glucose only increased modestly from baseline after lunch and after dinner (Figure 3C). The daylong average concentration for glucose was higher for the PI group than for the NNRTI group (111 ± 9 versus 103 ± 10 mg/dL), although the difference was not significant (P = 0.06). No significant difference in the incremental postprandial daylong average for glucose was found.

**DISCUSSION**

In the present study, we evaluated whether baseline concentrations predicted postprandial responses to a physiologic caloric load in 25 HIV-infected African American and Hispanic subjects without hyperlipidemia (total cholesterol and triacylglycerol concentrations < 200 mg/dL) and receiving stable antiretroviral regimens and whether the postprandial response was modulated by PI-based or NNRTI-based ART. We also compared baseline variables for the HIV-positive patients with those of a normal, healthy population and the postprandial triacylglycerol response to results obtained previously in HIV-negative subjects with signs of insulin resistance.

The results showed that although the HIV population investigated had no signs of hyperlipidemia at baseline, the HIV-infected patients had lower HDL-cholesterol concentrations than did healthy, population-based control subjects matched for age, sex, ethnicity, and BMI. Baseline total and LDL-cholesterol, triacylglycerol, and apo B concentrations were significant, positive predictors of the postlunch postprandial incremental triacylglycerol response, whereas HDL-cholesterol concentrations negatively predicted the same response. Within the HIV group, fasting triacylglycerol and insulin concentrations and the postprandial triacylglycerol AUC were higher among PI-treated patients than in NNRTI-treated patients. However, the incremental triacylglycerol response was similar between the 2 ART groups.

Most studies of postprandial responses to a test meal to date have measured the response to a standardized, high-caloric meal or fat-rich shake, which is usually given in the morning (28–30). Although this approach is useful in addressing clearing mechanisms of postprandial lipoproteins and the possible accumulation of remnant lipoproteins, it does not reflect the common eating pattern. The diet intake pattern used in the present study, however, reflects the recommended eating pattern for free-living individuals. The response to the physiologic caloric load in the

**TABLE 6**

<table>
<thead>
<tr>
<th>Variable</th>
<th>PI-based ART</th>
<th>NNRTI-based ART</th>
<th>PI-based ART</th>
<th>NNRTI-based ART</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacylglycerols, 0–12 h²</td>
<td>205 (186–248)</td>
<td>125 (78–191)³</td>
<td>42.3 (29–93)</td>
<td>37.0 (16–80)</td>
</tr>
<tr>
<td>Glucose, 0–12 h³</td>
<td>111 ± 9</td>
<td>103 ± 10</td>
<td>3.6 ± 18</td>
<td>5.4 ± 6</td>
</tr>
<tr>
<td>Insulin, 0–12 h³</td>
<td>61.7 (34–93)</td>
<td>37.7 (20–53)</td>
<td>9.0 (3.2–25)</td>
<td>6.0 (2.5–13)</td>
</tr>
</tbody>
</table>

¹ n = 13 for the PI group and n = 12 for the NNRTI group. PI, protease inhibitor; ART, antiretroviral therapy; NNRTI, nonnucleoside reverse transcriptase inhibitor.
² All values for triacylglycerols are median; interquartile range in parentheses.
³ Significantly different from PI group. P < 0.05 ( t test).
⁴ All values for glucose are x ± SD.
⁵ All values for insulin are x; ± 1 SD in parentheses.
the day with a muted early postprandial triacylglycerol increase. In contrast, the glucose response after a meal was greater in the morning. Because the degree of infectious burden during HIV may affect key metabolic pathways (9, 43–45), it is possible that this late triacylglycerol response may be influenced by HIV status. To address this possibility, we performed an exploratory analysis using historical data from HIV-negative control subjects who participated in the DELTA Study. Although we did not carry out a direct parallel study in HIV-positive and HIV-negative subjects, the 2 groups (HIV-positive and HIV-negative subjects) shared key metabolic features, which provided an opportunity to compare results across HIV status. In addition, the HIV-positive and HIV-negative subjects were given the same type of diet, which was based on the AHA Step I diet. As shown in Figure 2, the triacylglycerol response was significantly more delayed for HIV-positive than for HIV-negative subjects, irrespective of ART regimen. This may suggest that HIV status modulates the degree of postprandial lipid exposure. However, further studies are needed to explore this possibility.

In previous studies, higher HDL-cholesterol concentrations were reported for persons receiving NNRTI-based ART than for those receiving PI-based ART (35). Although we did not find any such differences in the present study, which was likely due to the relatively small number of subjects enrolled and the exclusion of subjects with hyperlipidemia, PI-treated subjects had higher fasting triglyceride concentrations than did NNRTI-treated subjects. However, we noted no significant differences between the 2 HIV-positive groups with regard to postprandial triglyceride, insulin, or glucose responses. These findings indicate that after a physiologic caloric load based on the AHA Step I diet, postprandial concentrations of these key metabolic markers are not affected by the choice of ART regimen in subjects without fasting hyperlipidemia. Therefore, our findings may suggest that the activity of the lipid clearance pathways might be sufficient to accommodate the repeated physiologic meal challenges in normolipidemic ART-treated subjects, irrespective of PI- or NNRTI-based therapy. Although the incremental postprandial increase did not differ significantly between the 2 treatment groups, PI-treated subjects were exposed to a higher postprandial triglyceride concentration. Further studies are needed to explore whether such exposure could confer an increased cardiovascular disease risk.

We recognize that our study has some limitations. The number of participants was limited, and because all HIV-infected patients were receiving antiretroviral regimens, it is difficult to differentiate the effects of HIV status and those of ART. In addition, the recruitment of normolipidemic subjects limited our ability to address postprandial lipid concentrations in HIV-infected patients with fasting hyperlipidemia, a group likely prone to an enhanced postprandial response. Furthermore, we recruited African American and Hispanic patients. We recognize that our results need to be interpreted with these limitations in mind. However, the study also offers some strengths. We evaluated the detailed response to a postprandial challenge in HIV-infected patients, an understudied area, which allowed us to identify predictors of this response. Furthermore, this is one of the first studies addressing the effects of a physiologic caloric load in this group of patients.

In summary, we showed that baseline lipid variables predicted triacylglycerol response to a physiologic caloric load in HIV-positive subjects without hyperlipidemia who were receiving ART with either PI- or NNRTI-containing regimens. Furthermore, the response was similar in patients treated with PI-based or NNRTI-based ART. This finding suggests that in a physiologic setting and under normolipidemic conditions, choice of ART regimen may not be a major modulator of postprandial lipid metabolism; however, larger studies are needed to confirm this result. The slow postprandial increase in triglyceride concentrations for both groups suggests that HIV disease or its treatment may modulate postprandial triglyceride metabolism. To further assess whether postprandial lipemia would differ depending on antiretroviral regimen in response to a more pronounced dietary challenge, studies using a standardized, high-calorie challenge are warranted.

All authors contributed to the study design. The following authors contributed to the data collection (AT-G, SR, RM, BO, WK, JA, and LB), data analysis (SH, RR, HNG, WME-S, and LB), and manuscript preparation (AT-G, SH, RR, JA, HNG, WME-S, and LB). None of the authors had a conflict of interest to disclose.

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Alcohol intake and methylenetetrahydrofolate reductase polymorphism modify the relation of folate intake to plasma homocysteine

Stephanie E Chiuve, Edward L Giovannucci, Susan E Hankinson, David J Hunter, Meir J Stampfer, Walter C Willett, and Eric B Rimm

ABSTRACT

Background: Folate intake increases plasma folate and reduces total homocysteine (tHcy) concentrations, which may lower coronary artery disease (CAD) and cancer risks. Folate metabolism may be altered by alcohol intake and 2 common polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene, 677C→T and 1298A→C.

Objective: We examined whether the associations between folate intake and plasma folate and tHcy concentrations were modified by alcohol intake or variations in the MTHFR gene.

Design: We conducted a cross-sectional analysis among 988 women by using multivariate linear regression models to estimate mean plasma tHcy and folate concentrations. Folate intake was the sum of food and supplemental sources.

Results: We observed an inverse association between folate intake and tHcy, which was modified by alcohol intake (P for interaction = 0.04) and MTHFR677 genotype (P for interaction = 0.05) but not by MTHFR1298 genotype (P for interaction = 0.97). In the lowest quintile of folate intake, moderate drinkers (≥15 g alcohol/d) had significantly higher tHcy concentrations (15.2 ± 2.9 nmol/mL) than did light drinkers (11.3 ± 0.7 nmol/mL) and nondrinkers (11.0 ± 0.8 nmol/mL). However, the reduction in tHcy between the highest and lowest quintiles of folate intake was significantly greater in moderate drinkers (−6.6 nmol/mL) than in light drinkers (−2.3 nmol/mL) and nondrinkers (−2.1 nmol/mL). The elevated tHcy in women with low folate intake who also consumed moderate amounts of alcohol was even higher (22.4 ± 4.8 nmol/mL) in the presence of the variant MTHFR677 allele. The positive association between folate intake and plasma folate was somewhat modified by alcohol intake (P for interaction = 0.08) but not by either MTHFR genotype.

Conclusions: Moderate alcohol intake and low MTHFR activity have adverse effects on tHcy, but those effects may be overcome by sufficient folate intake.

KEY WORDS Folate intake, homocysteine, plasma folate, methylenetetrahydrofolate reductase, MTHFR, polymorphism, alcohol

INTRODUCTION

High concentrations of circulating total homocysteine (tHcy), a sulfur-containing amino acid, are associated with increased risk of coronary artery disease (CAD) (1). High tHcy concentrations are also an indicator of reduced DNA methylation, which may be a risk factor for some cancers (2). Homocysteine can be catabolized by a vitamin B-6–dependent pathway or remethylated to methionine by a pathway that requires folate, vitamin B-12, and riboflavin (Figure 1). Folic acid supplementation lowers tHcy in a linear fashion up to ≈400 μg/d, but tHcy concentrations plateau at higher doses (3, 4). The positive linear association between folate intake and plasma folate extends to folic acid supplementation of >400 μg/d (3, 5).

Polymorphisms in the gene that encodes the methylenetetrahydrofolate reductase (MTHFR) enzyme may impede homocysteine remethylation. MTHFR irreversibly converts 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MTHF), the most prevalent form of folate in circulation (Figure 1). A C→T transition at base pair 677, which causes an alanine-to-valine substitution, is associated with low enzyme activity (6). Heterozygotes (CT) and homozygotes (TT) have 71% and 33%, respectively, the activity of persons with the wild-type (CC) genotype (7). Persons with the TT genotype have significantly lower plasma folate (8) and higher tHcy concentrations (9, 10) than do those with the CC genotype. Although folate effectively decreases tHcy among all genotypes, persons with the TT genotype may require higher intakes than those with other genotypes to optimally reduce tHcy (11). A second variation at base pair 1298 (A→C) also results in decreased enzyme activity, and, although this polymorphism alone may not alter plasma folate or tHcy, combined heterozygosity for MTHFR677 and MTHFR1298 may significantly influence these plasma markers (12).

Alcohol (ethanol) can interfere with folate metabolism, either directly (13) or through its metabolite acetaldehyde (14, 15). The

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The concentration is twice as high, and plasma B vitamins are lower, among chronic alcoholics than among healthy controls, most likely as a result of a combination of malnourishment and the direct effects of heavy alcohol intake on folate status (16). The effect of moderate alcohol intake on homocysteine metabolism is unclear. Previous studies have reported positive (17–20) and inverse (21–24) associations between alcohol intake and tHcy. Our objective in this study was to assess whether the associations of folate intake with fasting plasma folate and tHcy concentrations were modified by MTHFR genotype or alcohol intake among women.

SUBJECTS AND METHODS

Study population

The Nurses’ Health Study

The Nurses’ Health Study (NHS) is a prospective cohort consisting of 121,700 female nurses aged 30–55 y at baseline in 1976. Participants provided medical history and lifestyle information on mailed questionnaires at the study’s inception and biennially thereafter. Blood samples were collected from 32,826 women in 1989–1990. As described previously (25), the women received a kit that contained necessary supplies for venipuncture. The women returned the whole-blood samples in an enclosed ice pack via overnight mail, along with a completed short questionnaire that obtained information on menopausal status, recent postmenopausal hormone use, the time since the last meal, and the time of day of the blood drawing. On arrival, samples were centrifuged, separated, and stored in the gas phase of a liquid nitrogen freezer at −130 °C. The women in this analysis were healthy control subjects, not currently using exogenous hormones, from separate nested case-control studies of cardiovascular disease and colon neoplasia (26). Participants had no history of cancer (except nonmelanoma skin cancer), stroke, myocardial infarction, angina, or revascularization surgery before they returned their blood samples.

The Nurses’ Health Study II

The NHS II (NHS2) is a prospective cohort of 116,671 female nurses aged 25–41 y at baseline in 1989. Blood samples were obtained in 1997 and 1998 from 29,613 women. The methods used to obtain lifestyle and medical information and blood samples were similar to those described above.

We analyzed blood from a subset of premenopausal women who were not users of exogenous hormones. For this sample collection, women were in the luteal phase of their menstrual cycle. Women with a history of cardiovascular disease, diabetes mellitus, gastric or duodenal ulcers, liver or gallbladder disease, or cancer (excluding nonmelanoma skin cancer) before date of blood draw were excluded. From the remaining women, we randomly selected 473 on the basis of self-reported alcohol use to study the effects of alcohol on biological markers of CAD. Specific drinking patterns determined on the basis of frequency, amount, and use with meals were oversampled for adequate variation. The characteristics of this population did not differ significantly from those of the larger cohort. Further details on the selection process were published elsewhere (27).

Written informed consent was obtained from all participants. The Institutional Review Board of the Harvard School of Public Health approved the study protocol.

Assessment of dietary and nondietary factors

We assessed dietary information with a validated semi-quantitative food-frequency questionnaire (FFQ). Average nutrient intake over the previous year was calculated from the FFQ by using nutrient values obtained from the Harvard University Food Composition Database, which was derived from the US Department of Agriculture and other sources. The reproducibility and validity of the FFQ have been documented elsewhere (28, 29). The correlations between the FFQ and multiple 1-wk diet records were 0.88 for riboflavin, 0.85 for vitamin B-6, 0.56 for vitamin B-12, 0.77 for folate, and 0.84 for alcohol intake. All

FIGURE I. Homocysteine metabolism by transulfuration and remethylation. Homocysteine can be converted to cysteine by a vitamin B-6–dependent transulfuration. Homocysteine can also be remethylated to methionine through a vitamin B-12–and folate-dependent pathway. tHcy, total homocysteine; THF, tetrahydrofolate; DHF, dihydrofolate; 5,10-MTHF, 5,10-methylene THF; 5-MTHF, 5-methyl THF; MTHFR, methylenetetrahydrofolate reductase gene; MS, methionine synthase.
nutrient intakes were adjusted for total energy by using the residual method (30). Total intake for all vitamins, including folate, was the sum of food and supplemental sources.

We used the 1990 FFQ for NHS and the 1999 FFQ for NHS2 to correspond to blood samples drawn in 1989–1990 and 1997–1998, respectively, because the FFQs assess diet over the previous year. The food-composition database was updated to reflect the fortification of grain products with folate, which began in 1996 and became mandatory in 1998 (31). Information on systolic blood pressure, use of aspirin and hypertensive medication, smoking status, physical activity, height, and weight was obtained through self-administered questionnaires, and we used the questionnaire that was completed the closest in time to the blood drawing.

Assays for plasma markers

Because this analysis was based on samples from several different datasets, some analytes were measured with different methods. We controlled for laboratory batch in our analysis. For samples from the NHS, plasma folate was measured by using a radioimmunoassay kit (Bio-Rad, Richmond, CA), and tHcy was measured by using HPLC at the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging (Tufts University, Boston, MA). For the NHS2 samples, both plasma folate and tHcy concentrations were measured by using an immunoassay on an IMx analyzer (Abbott Laboratories, Abbott Park, IL) at the laboratory of Nadar Rifai (Children’s Hospital, Boston MA). Quality-control samples (5% of all samples), obtained from a plasma pool from healthy volunteers, were given given indicator identification numbers and interspersed randomly among the specimens. The CVs were <10% for plasma folate and tHcy.

DNA was extracted from buffy coat fractions, and MTHFR677 and MTHFR1298 genotypes were assessed by using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probes are available from the authors on request. Polymerase chain reaction amplifications were carried out on 5–20 ng DNA using 1× TaqMan universal PCR master mix (Applied Biosystems). Amplification conditions were 1 cycle of 95 °C for 10 min and then 50 cycles of 92 °C for 15 s and 58 °C for 1 min. The frequencies of both genotypes were in Hardy-Weinberg equilibrium.

Exclusions

Women whose information on folate intake or smoking status was missing and those who did not fast ≥6 h before the blood drawing were excluded from all analyses. Women whose concentrations of plasma folate or tHcy or information on alcohol intake or MTHFR genotype was missing were excluded only from analyses that required these specific variables. The total population consisted of 1146 women. Of these women, 988 were included in the homocysteine analysis and 966 in the plasma folate analysis.

Statistical analysis

We used multivariate linear regression to calculate mean concentrations of plasma tHcy and folate in each quintile of folate intake. Robust variance estimates were used for valid statistical inference of linear regression models, despite a lack of normality in the outcome variable (32). In multivariate models, we adjusted for age (centered at 50 y); smoking status (never or past, current <15 cigarettes/d, or current ≥15 cigarettes/d); regular aspirin use (yes or no); hypertensive medication use (yes or no); intakes of riboflavin, vitamins B-6 and B-12 (centered at median), methionine (above or below median of 1.9 g/d), and coffee (cups/d); laboratory batch; MTHFR genotype (CC, CT, or TT); and alcohol intake (0 g/d, 0.01–14.9 g/d, or ≥15.0 g/d). Further adjustment for tea intake, physical activity, body mass index, total dietary protein, and dietary fat intake had no appreciable effect on results (data not shown). To conduct a test for trend, we created a continuous score variable for folate intake by using the median value from each quintile.

We stratified the predictive models for plasma tHcy and folate by MTHFR677 genotype, assuming a codominant mode of inheritance (CC, CT, and TT). To test formally for interaction, we included in our models the multiplicative interaction term of folate intake (as a continuous variable, for which the median value for each category of folate intake was used) and MTHFR677 genotype (as a continuous variable with 3 categories). We performed similar analyses that were stratified by MTHFR1298 genotype (AA, AC, and CC) and alcohol intake (0 g/d, 0.01–14.9 g/d, and ≥15 g/d). To evaluate the interaction between alcohol intake and MTHFR677 genotype, we stratified models of plasma tHcy and folate by alcohol, separately in women with the CC genotype and in women with at least one copy of the variant allele. We had limited power to examine this interaction among women with the TT genotype alone because of the limited number of participants with this genotype (n = 34, 60, and 15 for nondrinkers, light drinkers, and moderate drinkers, respectively). Therefore, we combined women with CT and TT genotypes. We included the multiplicative interaction term of alcohol and MTHFR677 genotype (CC or CT/TT) in our models. We performed a similar analysis with alcohol and the MTHFR1298 genotype. All P values are two-tailed. Statistical analyses were conducted with SAS software (version 8; SAS Institute, Cary, NC).

RESULTS

The age-adjusted characteristics of the population used in the homocysteine analysis are shown by quintile of folate intake in Table 1. The distribution of these characteristics did not differ significantly within the population for the plasma folate analysis (data not shown). The median folate intake of women in the lowest quintile was 241 μg/d. Women with high folate intake tended to be younger than those with low folate intake. More women from the NHS2 than from the NHS contributed to the higher folate quintiles because blood and dietary information were collected from the former group after the fortification of grain products. As expected, the intake of other B vitamins increased as folate intake increased. The average alcohol intake did not differ significantly across quintiles of folate intake. Except for the CT variant of MTHFR677, frequencies of the MTHFR677 and MTHFR1298 genotypes did not differ across quintiles of folate intake. The 2 polymorphisms were in linkage disequilibrium; among the women with the TT genotype for MTHFR677, only 1 carried the variant allele for the MTHFR1298 polymorphism, and none of the women who were homozygous for a variant of MTHFR1298 did so. Folate intake was inversely associated with plasma tHcy
and positively associated with plasma folate in both age-adjusted and multivariate models (Table 2).

**Modification of the folate intake–tHcy relation by MTHFR genotypes and alcohol intake**

The MTHFR677 polymorphism modified the dose-response association between folate intake and tHcy (P for interaction = 0.05) (Figure 2). The inverse association between folate intake and tHcy was strongest among the women with the variant allele. We observed a reduction in mean tHcy from 13.2 nmol/mL in the first quintile to 8.8 nmol/mL in the fifth quintile among women who were heterozygous for the CT variant and from 11.6 to 8.3 nmol/mL among those who were homozygous for the TT variant. We observed the smallest reduction in tHcy (from 10.9 to 9.3 nmol/mL) between the lowest and highest quintiles of folate intake among women who had the wild-type MTHFR677 genotype (CC). We did not observe an interaction between folate intake and the MTHFR1298 genotype for tHcy (P for interaction = 0.97).

Compound heterozygosity (CT677/AC1298) was not associated with a greater response to folate intake. Among women who were heterozygous for the CT variant of MTHFR677, the trend in mean tHcy across quintiles of folate intake did not differ significantly between women who had the wild-type AA variant and those who were heterozygous for the AC variant of MTHFR1298 (P for interaction = 0.38).

Alcohol intake significantly modified the association between folate intake and tHcy (P for interaction = 0.04) (Figure 3). Higher folate intake was only modestly associated with lower tHcy among light drinkers and nondrinkers, whereas, among moderate drinkers, this inverse relation was much stronger. Among moderate drinkers, we observed a reduction in mean tHcy from 15.2 nmol/mL in the first quintile to 8.9 nmol/mL in the fifth quintile; the decrease in tHcy from the first quintile to the fifth quintile of folate intake was from 11.3 to 9.0 nmol/mL among light drinkers and from 11.0 to 8.9 nmol/mL among nondrinkers. The steepest decline in tHcy among the moderate drinkers was seen with low folate intake (quintiles 1 and 2), whereas the dose-response curve of moderate drinkers began to approximate the curves of light drinkers and nondrinkers in the third quintile of folate intake (≈400 µg/d).
Women with heavy alcohol intake may have a different dose response between folate intake and tHcy, as well as a different set of potential confounders of the dose response, than do moderate drinkers. To remove this extraneous variation, in a secondary analysis, we excluded women who drank $>50$ g alcohol/d ($n = 11$). However, results from this analysis were not appreciably different (data not shown).

The inverse association between folate intake and tHcy among the moderate alcohol drinkers was primarily limited to the women with $\geq 1$ variant allele for MTHFR677 (alcohol $\times$ genotype interaction, $P = 0.01$) (Table 3). Although the moderate drinkers with the variant allele had elevated tHcy (22.4 nmol/L at low folate intake, tHcy was no longer elevated with high folate intake, especially when the heaviest drinkers ($\geq50$ g alcohol/d; $n = 11$) were excluded from the analysis. The MTHFR1298 $\times$ alcohol interaction for tHcy was not significant ($P = 0.72$).

### Modification of the folate intake–plasma folate relation by MTHFR genotypes and alcohol intake

The relation between folate intake and plasma folate was not modified by the MTHFR677 ($P$ for interaction = 0.97) or MTHFR1298 ($P$ for interaction = 0.13) genotype. This relation
TABLE 3
Adjusted mean values of total homocysteine (tHcy) by alcohol consumption and MTHFR677 genotype across quintiles of energy-adjusted folate intake

<table>
<thead>
<tr>
<th>Plasma tHcy</th>
<th>Quintiles of folate intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median folate intake (µg/d)</td>
<td>1</td>
</tr>
<tr>
<td>0 g alcohol/d</td>
<td>241</td>
</tr>
<tr>
<td>CC (n = 121)</td>
<td>10.4 ± 1.3</td>
</tr>
<tr>
<td>0.01–14.9 g alcohol/d</td>
<td>10.9 ± 1.0</td>
</tr>
<tr>
<td>CT (n = 232)</td>
<td>12.3 ± 1.0</td>
</tr>
<tr>
<td>TT (n = 263)</td>
<td>11.9 ± 1.0</td>
</tr>
<tr>
<td>≥15.0 g alcohol/d</td>
<td>6.2 ± 0.9</td>
</tr>
<tr>
<td>CC (n = 66)</td>
<td>22.4 ± 4.8</td>
</tr>
<tr>
<td>CT (n = 91)</td>
<td>6.3 ± 1.7</td>
</tr>
<tr>
<td>TT (n = 84)</td>
<td>18.6 ± 3.9</td>
</tr>
</tbody>
</table>

1 Linear regression models were adjusted for age; intakes of riboflavin, vitamins B-6 and B-12, methionine, and coffee; smoking status; regular aspirin use; hypertension medication use; and laboratory batch. Interaction between alcohol (in 3 categories) and methylenetetrahydrofolate reductase (MTHFR) (in 2 categories), P = 0.01 for tHcy; folate (continuous variable based on median value of quintiles) × MTHFR interaction, P = 0.05; folate × alcohol intake interaction, P = 0.04; and alcohol × MTHFR × folate interaction, P = 0.89. 
2 Linear regression coefficient for continuous variables was based on median values from each quintile.
3 SE (all such values).
4,5 Significantly different from quartile 1 within the same category of alcohol and MTHFR677 genotype in the linear regression models (Wald test): P < 0.05. 6, P < 0.01.
7 The heaviest drinkers (≥50 g alcohol/d) were excluded.

was influenced by alcohol, although the interaction was not significant (P = 0.08) (Figure 4); the overall positive association was strongest among nondrinkers. We did not see further modification by variations in either the MTHFR677 (alcohol × genotype interaction, P = 0.49) or MTHFR1298 (alcohol × genotype interaction, P = 0.97) genotype.

DISCUSSION

Folate intake was inversely associated with fasting plasma tHcy, and this relation was modified by MTHFR677 genotype and alcohol intake. Women with the thermolabile variant of MTHFR677 or moderate alcohol intake had significantly higher tHcy at low intakes of folate. However, adequate folate intake minimized these differences. In addition, the elevation in tHcy among women who consumed low folate and drank moderate amounts of alcohol was greater in the presence of the variant MTHFR677 allele.

To our knowledge, this is the first study to examine the associations of the combination of folate intake, alcohol intake, and the MTHFR polymorphisms with plasma tHcy and folate. In previous studies, the association between alcohol intake and tHcy has been unclear (17–24), possibly because of differences in folate intake or a lack of stratification by folate and MTHFR genotype. The increase in tHcy among moderate drinkers with low intake of folate was restricted to women with the thermolabile variant of MTHFR. In women who had the wild-type MTHFR677 variant, tHcy concentrations were not significantly elevated among moderate alcohol drinkers.

Ideally, we would have examined separately the modification by alcohol intake of the relation between folate intake and both tHcy and plasma folate in the 3 MTHFR677 genotypes. Conventionally, persons with the CT genotype are combined with those who have the CC rather than the TT genotype (10, 33). However, because of the limited number of women who were homozygous for the variant allele, we were unable to examine any associations in these women separately. By combining subjects with the CT and TT genotypes, we could assess the combined effect of the variant allele and alcohol intake on these relations.

Significant interactions between alcohol intake, MTHFR677 genotype, and folate status have also been seen in studies of
CAD. The MTHFR677 polymorphism is an independent risk factor for CAD only among those with low folate status (34). Women with both high folate intake and moderate alcohol intake had a significantly lower risk of CAD than did nondrinkers with low folate intake (35). The effect of these interactions between folate intake, alcohol intake, and MTHFR genotype on the risk of CAD may be partially mediated through tHcy.

The evidence of an interaction between alcohol intake, MTHFR677 genotype, and folate status is equally strong with respect to cancer. Alcohol intake significantly increases the risk of breast (36, 37) and colon (38) cancer, although the association appears limited primarily to persons with low folate intake. The MTHFR677 polymorphism has been associated with reduced risk of colon cancer, but only in combination with a methyl-rich diet (low alcohol or high folate intake or both) (39). Persons with the variant MTHFR677 genotype may be more susceptible to colon cancer if they have a methyl-poor diet (ie, high alcohol or low folate intake or both). These interactions in relation to cancer are not likely to be due to a biological effect of homocysteine but rather to the importance of folate on gene methylation. A methyl-poor diet or reduced MTHFR activity may enhance carcinogenesis through a reduction in the 5-MTHF form of folate and a subsequent inhibition of homocysteine remethylation (Figure 1). Reduced remethylation could result, in addition to an accumulation of tHcy, in a decrease in methionine, which is used in DNA methylation. The markedly higher tHcy concentrations we observed in the methyl-poor subgroup in our study may reflect aberrations in DNA methylation, a potential risk factor for cancer (2).

Alcohol may interfere with folate metabolism through a reduction in folate absorption at the brush border (40) or an inhibition of the methionine synthase enzyme, which is needed to transfer a methyl group from 5-MTHF to homocysteine (41). The inhibition of this enzyme by alcohol traps folate in its 5-MTHF form (13) and may result in a 5-MTHF pool that cannot remethylate homocysteine (Figure 1). Inhibition of methionine synthase may be overcome by adequate plasma folate, achieved through sufficient folate intake or with efficient MTHFR activity, such as that among those who are wild-type.

As had earlier investigators (42), we found the MTHFR677 polymorphism to be a stronger determinant of tHcy than was the MTHFR1298 polymorphism. Combined heterozygosity of MTHFR677 and MTHFR1298 has been associated with higher tHcy concentrations than were seen with the MTHFR677 variant alone (43, 44). However, we did not observe a stronger association between folate intake and tHcy in those subjects with combined heterozygosity than in those with only the MTHFR677 variation.

Betaine, derived from choline, may lower tHcy independent of the folate remethylation pathway (45), especially in the presence of ethanol (46). Moreover, its metabolism may be influenced by MTHFR genotype (47). We were unable to assess betaine or other factors that may influence tHcy and contribute to its variability. Unless correlated with folate intake, these factors would not affect the magnitude of the association between folate intake and tHcy.

Others have reported an interaction between folate status and alcohol intake in relation to tHcy, but only among men (48), which may be due to the larger variation in alcohol intake among men than among women. To increase variation in alcohol intake, we oversampled the drinkers in the NHS2 population. Because blood was drawn both before and after folate fortification, we also have a wider range of folate intakes than had been previously studied. The median of our lowest quintile of folate intake (241 μg/d) may still be high with respect to that in other populations, especially those without fortification programs. The elevation of tHcy at low folate intake among alcohol drinkers or among persons with the MTHFR variant may be even greater in other populations.

Although everyone should consume sufficient folate, these findings specifically highlight the necessity of adequate folate intake among moderate drinkers. In our study, when all groups had a folate intake of ≥400 μg/d, tHcy concentrations among moderate drinkers began to approach those among light drinkers and nondrinkers. Alcohol drinkers who have the variant MTHFR677 allele may require even higher folate intakes.

We observed that, among moderate drinkers, tHcy was no longer elevated with high folate intake. We observed this relation only after excluding heavy alcohol consumers (≥50 g/d) from the analysis. This finding suggests that, whereas the adverse effects of moderate alcohol may be overcome through adequate folate intake, the same may not be true with excessive intake of alcohol.

In conclusion, concentrations of plasma folate and tHcy are primarily determined by the intake of folate. However, alcohol intake and genetically determined MTHFR activity can modify these dose-response associations. Future research on the health effects of folate intake on tHcy, CAD, or cancer should incorporate these potential risk modifiers.

We thank the participants of the NHS and NHS2 for their cooperation and participation.

SEC was responsible for the design of the study, analysis of the data, and writing the manuscript; ELG, SEH, DJH, MJS, and WCW were responsible for critical review of the manuscript. EBR was responsible for securing funding, design of the study, analysis of the data, and writing the manuscript. No authors had any financial or personal interest in any organizations sponsoring the research reported in this article.

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Diet-quality scores and plasma concentrations of markers of inflammation and endothelial dysfunction

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ABSTRACT

Background: Endothelial dysfunction is one of the mechanisms linked to an increased risk of cardiovascular disease.

Objective: We assessed the association between several diet-quality scores and plasma concentrations of markers of inflammation and endothelial dysfunction.

Design: Diet-quality scores on the Healthy Eating Index (HEI), Alternate Healthy Eating Index (AHEI), Diet Quality Index Revised (DQI-R), Recommended Food Score (RFS), and the alternate Mediterranean Diet Index (aMED) were calculated by using a food-frequency questionnaire that was administered in 1990 to 690 women in the Nurses’ Health Study (ages 43–69 y, no cardiovascular disease or diabetes). Blood collection was completed in the same year. We used regression analysis to assess the associations between these diet-quality scores and plasma concentrations of C-reactive protein, interleukin 6, E-selectin, soluble intercellular adhesion molecule 1, and soluble vascular cell adhesion molecule 1.

Results: The various diet-quality scores were significantly correlated with each other; correlation coefficients ranged from 0.56 to 0.80 (all \( P \) values < 0.0001). After adjustment for age, body mass index, alcohol intake, physical activity, smoking status, and energy intake, the HEI and DQI-R were not significantly associated with any of the biomarkers, whereas the AHEI and aMED scores were associated with significantly lower concentrations of most biomarkers. The RFS was significantly associated with a lower concentration of E-selectin only. C-reactive protein concentrations were 30% (\( P < 0.05 \)) and 24% (\( P < 0.05 \)) lower in the top than in the bottom quintile of the AHEI and of the aMED, respectively.

Conclusion: Higher AHEI and aMED scores were associated with lower concentrations of biomarkers of inflammation and endothelial dysfunction and therefore may be useful as guidelines for reducing the risk of diseases involving such biological pathways. Am J Clin Nutr 2005;82:163–73.

KEY WORDS: Diet, diet quality, inflammation, endothelial dysfunction, women

INTRODUCTION

Endothelial dysfunction and inflammation are believed to be involved in diseases such as atherosclerosis and diabetes (1, 2). High concentrations of markers of inflammation and endothelial dysfunction, such as C-reactive protein (CRP), interleukin 6 (IL-6), E-selectin, and soluble intercellular adhesion molecule 1 (sICAM-1), have been shown to predict cardiovascular disease risk (3–5). Data are also emerging about their involvement in the development of diabetes. A recent study showed a positive association between serum concentrations of sICAM-1 and E-selectin and risk of diabetes (6). The associations between foods, nutrients, and these diseases may be mediated in part through inflammation and endothelial dysfunction. Studies have suggested a link between dietary intake of long-chain n−3 fatty acids and antioxidants and endothelial dysfunction (7) and of alcohol (8), vitamin B-6 intake, and glycemic index (9) and plasma CRP concentrations (10). However, data are scant on the association between overall dietary patterns and these biomarkers.

Several indexes to assess overall diet quality have been proposed. The Healthy Eating Index (HEI) developed by the US Department of Agriculture was based on the Dietary Guidelines for Americans and the Food Guide Pyramid (11). The Diet Quality Index Revised (DQI-R) is based on similar guidelines from the National Research Council but also includes iron and calcium (12). The Recommended Food Score (RFS) was constructed from foods recommended from the current intake guidelines (13). Our group previously revised the HEI according to the most recent scientific evidence to focus on the healthier items in the food guide pyramid food groups (14). This Alternate Healthy Eating Index (AHEI) was found to be better than the original HEI or RFS at predicting the risk of cardiovascular disease and the overall incidence of major chronic diseases. Mortality and rates

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2 Supported by NIH grants CA87969 and DK58845. FBH was a recipient of the American Heart Association Established Investigator Award.

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of several chronic diseases are lower in the Mediterranean region, and the traditional regional diet is believed to play a role in this lower risk (15). Trichopoulou et al (16) developed a scale to quantify this diet in the Greek population. We adapted this to develop the alternate Mediterranean Diet Score (aMED) to use with a food-frequency questionnaire (FFQ) developed in the United States.

Because the purpose of these diet-quality indexes is to assess and guide an individual’s dietary intake for the promotion of health and prevention of disease, they need to be examined for their utility. One approach is to assess and compare how well these measures of diet quality relate to biomarkers of disease risk or disease endpoints. In this analysis, we focused on the relation between these diet-quality indexes and biomarkers of inflammation and endothelial dysfunction. Specifically, we assessed the associations between the HEI score, an alternate HEI score, the RFS, the DQI-R, and the aMED and plasma concentrations of C-reactive protein (CRP), interleukin 6 (IL-6), E-selectin, soluble intercellular adhesion molecule 1 (sICAM-1), and soluble vascular cell adhesion molecule 1 (sVCAM-1).

SUBJECTS AND METHODS

Subjects

The Nurses’ Health Study was established in 1976 with the enrollment of 121,700 female nurses in the United States. The study was approved by the Institutional Review Board of the Brigham and Women’s Hospital, Boston, MA. Every 2 y, questionnaires were sent to update health, medical, and lifestyle information. A validated, semiquantitative FFQ was sent every 4 y to collect information on dietary intake for the previous year. In this study, we included 690 nurses who were selected as control subjects in a previous nested case-control study of diabetes. These women were 43–69 y of age and had no history of cardiovascular disease, cancer, or diabetes at the time blood was drawn.

Blood collection and measurement of biomarkers

Blood was collected in 1989–1990. Each willing participant was sent a blood collection kit containing instructions and needed supplies (eg, blood tubes and needles). Each participant made arrangements for blood to be drawn, packaged the sample in an enclosed cool pack, and sent it to the laboratory by overnight courier. Almost all of the samples arrived within 26 h after blood was drawn. On arrival at the laboratory, the whole-blood samples were centrifuged (1200 × g, 15 min, room temperature) separated into aliquots, and stored at temperatures no higher than −80 °C. The lifestyles and dietary intakes of women who returned a blood sample were in general similar to those who did not provide a blood sample. All biomarkers were measured in the Clinical Chemistry Laboratory at the Children’s Hospital in Boston. CRP was measured with a latex-enhanced turbidimetric assay on a Hitachi 911 (Denka Seiken, Tokyo, Japan). IL-6 was measured with an ultrasensitive enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). E-selectin, sICAM-1, and sVCAM-1 were measured with a commercial enzyme-linked immunosorbent assay (R&D Systems). Interassay CVs were 3.4–3.8% for CRP, 5.8–8.2% for IL-6, 6.4–6.6% for E-selectin, 6.1–10.1% for sICAM-1, and 8.5–10.2% for sVCAM-1.

Assessment of dietary intake and diet-quality scores

The 1990 FFQ included ≈140 food items. A standard portion size was given for each food item. Cohort members were asked to choose from 9 possible frequency responses ranging from “never” to “≥6 times/d” for each food. Previous validation studies among members of the Nurses’ Health Study cohort showed good correlations between nutrients assessed with the FFQ and with multiple weeks of food records completed over the previous year (17, 18). Intake information from the FFQ was used to calculate the various diet-quality scores.

Calculation of the HEI was based on criteria set in The Healthy Eating Index Final Report (11) and adapted to this cohort by McCullough et al (19) (see Appendix A). Briefly, the HEI contains 10 components that reflect recommendations based on the Food Guide Pyramid (20) and the Dietary Guidelines for Americans (21). Recipe ingredients for mixed items were allocated to the appropriate food groups. Possible scores from each component ranged from 0 to 10, depending on level of intake, with a total possible score of 100 for the HEI. The AHEI scoring criteria (14) differ from those of the original HEI; more specific items, such as protein source, trans fat, ratio of polyunsaturated to saturated fat, and cereal fiber, are used in the AHEI rather than the broader terms, such as grains, total fats, and all meats combined, used in the HEI (see Appendix A). In addition, points were awarded for moderate alcohol consumption and long-term multivitamin use. The possible score for the multivitamin component was either 2.5 or 7.5 to avoid overweighting. The AHEI was based on 9 items, with a maximum possible score of 87.5.

The RFS was developed by Kant et al (13, 22) and adapted by McCullough et al (14) for our FFQ. The RFS focused on fruit, vegetables, whole grains, lean meats or meat alternates, and low-fat dairy products. Participants received 1 point for each recommended food consumed at least weekly. Based on the length of our FFQ, the maximum possible score was 51 (see Appendix A).

The DQI-R score was based on methods by Haines et al (12) and adapted for our FFQ by Newby et al (23) Briefly, the DQI-R consists of 10 components that measure intake of several food groups and nutrients and diet diversity and moderation. The range of possible scores for each component is 0–10 points, depending on the level of intake, and the maximum possible DQI-R score is 100 points.

The aMED score was based on the Mediterranean diet scale of Trichopoulou et al (16, 24). The original score was based on the intake of 9 items: vegetables, legumes, fruit and nuts, dairy, cereals, meat and meat products, fish, alcohol, and the ratio of monounsaturated to saturated fat. Intakes above the median of the study subjects received 1 point; all other intakes received 0 points. Meat and dairy product consumption less than the median received 1 point. We modified the original scale for this study by excluding potato products from the vegetable group, separating fruit and nuts into 2 groups, eliminating the dairy group, including whole-grain products only, including only red and processed meats for the meat group, and assigning alcohol intake between 5 and 15 g/d for 1 point. These modifications were based on dietary patterns and eating behaviors that have been consistently associated with lower risks of chronic disease in clinical and epidemiologic studies. Possible scores on the aMED ranged from 0 to 9 (see Appendix A).
TABLE 1

Age-standardized lifestyle characteristics by quintile (Q) of diet-quality scores

<table>
<thead>
<tr>
<th>Quintile of diet score</th>
<th>BMI</th>
<th>Current smoker</th>
<th>Activity</th>
<th>Energy intake</th>
<th>Total fat</th>
<th>Fiber</th>
<th>Glycemic load</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Eating Index</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q1 (36.0–66.4)</td>
<td>26.6 ± 6.8</td>
<td>27 ± 44</td>
<td>10 ± 13</td>
<td>1495 ± 626</td>
<td>62 ± 33</td>
<td>12.9 ± 5.2</td>
<td>121 ± 54</td>
</tr>
<tr>
<td>Q3 (74.6–80.5)</td>
<td>27.0 ± 5.9</td>
<td>18 ± 38</td>
<td>14 ± 15</td>
<td>1713 ± 518</td>
<td>60 ± 25</td>
<td>19.3 ± 8.0</td>
<td>160 ± 55</td>
</tr>
<tr>
<td>Q5 (86.6–98.7)</td>
<td>25.1 ± 5.2</td>
<td>5 ± 22</td>
<td>17 ± 19</td>
<td>2053 ± 379</td>
<td>63 ± 16</td>
<td>26.1 ± 7.7</td>
<td>212 ± 50</td>
</tr>
<tr>
<td>P for trend</td>
<td>0.05</td>
<td>&lt;0.0001</td>
<td>0.0005</td>
<td>&lt;0.0001</td>
<td>0.55</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Alternate Healthy Eating Index</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q1 (15.7–32.3)</td>
<td>27.5 ± 6.9</td>
<td>24 ± 43</td>
<td>10 ± 13</td>
<td>1559 ± 513</td>
<td>61 ± 26</td>
<td>13.4 ± 4.4</td>
<td>138 ± 51</td>
</tr>
<tr>
<td>Q3 (38.9–44.6)</td>
<td>25.7 ± 5.3</td>
<td>10 ± 30</td>
<td>13 ± 16</td>
<td>1848 ± 532</td>
<td>66 ± 26</td>
<td>19.7 ± 5.7</td>
<td>171 ± 53</td>
</tr>
<tr>
<td>Q5 (51.8–78.5)</td>
<td>25.1 ± 5.2</td>
<td>4 ± 19</td>
<td>16 ± 19</td>
<td>1975 ± 494</td>
<td>63 ± 22</td>
<td>27.7 ± 9.1</td>
<td>197 ± 64</td>
</tr>
<tr>
<td>P for trend</td>
<td>0.03</td>
<td>&lt;0.0001</td>
<td>0.004</td>
<td>&lt;0.0001</td>
<td>0.09</td>
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<td>Diet Quality Index Revised</td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Q1 (32.1–54.7)</td>
<td>26.8 ± 6.7</td>
<td>28 ± 45</td>
<td>9 ± 11</td>
<td>1608 ± 587</td>
<td>68 ± 30</td>
<td>13.7 ± 5.0</td>
<td>131 ± 52</td>
</tr>
<tr>
<td>Q3 (63.0–70.4)</td>
<td>27.0 ± 6.4</td>
<td>8 ± 28</td>
<td>14 ± 14</td>
<td>1799 ± 506</td>
<td>65 ± 24</td>
<td>19.6 ± 6.5</td>
<td>163 ± 52</td>
</tr>
<tr>
<td>Q5 (80.4–93.9)</td>
<td>25.2 ± 5.1</td>
<td>6 ± 23</td>
<td>16 ± 15</td>
<td>1901 ± 432</td>
<td>54 ± 16</td>
<td>26.3 ± 9.4</td>
<td>207 ± 62</td>
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<td>0.006</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<td>&lt;0.0001</td>
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<tr>
<td>Recommended Food Score</td>
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<td></td>
</tr>
<tr>
<td>Q1 (1–12)</td>
<td>26.2 ± 6.2</td>
<td>22 ± 42</td>
<td>9 ± 11</td>
<td>1536 ± 560</td>
<td>58 ± 28</td>
<td>13.0 ± 4.6</td>
<td>138 ± 61</td>
</tr>
<tr>
<td>Q3 (17–19)</td>
<td>26.1 ± 6.4</td>
<td>11 ± 32</td>
<td>14 ± 14</td>
<td>1792 ± 499</td>
<td>63 ± 22</td>
<td>20.0 ± 6.1</td>
<td>168 ± 59</td>
</tr>
<tr>
<td>Q5 (25–41)</td>
<td>27.0 ± 6.7</td>
<td>6 ± 24</td>
<td>15 ± 18</td>
<td>2099 ± 453</td>
<td>68 ± 23</td>
<td>27.4 ± 8.6</td>
<td>208 ± 55</td>
</tr>
<tr>
<td>P for trend</td>
<td>0.41</td>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0005</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<tr>
<td>Alternate Mediterranean Diet Index</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q1 (0–2)</td>
<td>27.1 ± 6.8</td>
<td>23 ± 42</td>
<td>9 ± 10</td>
<td>1533 ± 486</td>
<td>56 ± 22</td>
<td>13.2 ± 4.0</td>
<td>138 ± 52</td>
</tr>
<tr>
<td>Q3 (4)</td>
<td>26.3 ± 5.8</td>
<td>11 ± 32</td>
<td>15 ± 16</td>
<td>1742 ± 457</td>
<td>63 ± 23</td>
<td>19.2 ± 5.6</td>
<td>161 ± 52</td>
</tr>
<tr>
<td>Q5 (5)</td>
<td>26.5 ± 6.1</td>
<td>10 ± 29</td>
<td>15 ± 17</td>
<td>2033 ± 492</td>
<td>68 ± 24</td>
<td>26.8 ± 8.5</td>
<td>200 ± 58</td>
</tr>
<tr>
<td>P for trend</td>
<td>0.43</td>
<td>0.001</td>
<td>0.004</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

1 All values are x ± SD; n = 660.
2 Range in parentheses.
3 Walking and flights of stairs climbed; expressed as metabolic equivalent (METs) hours per week.

Assessment of other variables

Smoking status was self-reported in the 1990 main questionnaire. Body mass index (BMI) was calculated from the height reported in 1976 and the weight reported in 1990. Physical activity was expressed as hours of walking and flights of stairs climbed per week in 1990 and was converted to metabolic equivalent (METs) hours per week.

Statistical analysis

We calculated z scores for each of the diet-quality scores; log-transformation did not influence the results, so nontransformed data are presented. We then used a multivariate linear regression with a robust variance estimate (25) to examine associations between 1-z score increases in diet-quality score and biomarker concentrations. This variance estimator allows for valid inference without the assumption of normal distribution in the dependent variable. The regression models were adjusted for age (continuous), alcohol intake (nondrinker, 0.1–4.9 g, 5.0–9.9 g, ≥10 g/d), smoking status (never smokers, past smokers, current smokers of ≤14 cigarettes/d, and current smokers of >14 cigarettes/d), physical activity (<1.5, 1.5–5.9, 6–11.9, 12–20.9, ≥21 METs/wk), total energy intake (quintiles), and BMI (continuous). In addition, we also compared the geometric means of biomarker concentrations between women at the top and bottom quintiles for each diet-quality score. Then we compared this difference with t tests, including Bonferroni correction, between different diet-quality score.

RESULTS

The mean (±SD) diet-quality scores of our 660 participants were as follows: HEI = 77 ± 11, AHEI = 43 ± 11, DQI-R = 68 ± 13, RFS = 18 ± 7, and aMED = 4.4 ± 1.8. Mean (±SD) biomarker values were as follows: CRP = 2.8 ± 3.5 mg/L, IL-6 = 2.4 ± 3.8 ng/L, E-selectin = 48.8 ± 23.6 ng/L, sICAM-1 = 257 ± 78 μg/L, and sVCAM-1 = 547 ± 165 μg/L. The age-standardized lifestyle characteristics of the participants are shown in Table 1. Lower BMI was noted with higher scores of HEI, AHEI, and DQI-R. On all diet-quality indexes, individuals who scored high were less likely to be smokers and had higher levels of physical activity. Because a greater food intake may result in meeting consumption criteria of food items specified in various diet-quality scores, we observed that a higher

TABLE 2

Spearman’s correlation coefficients among diet-quality scores from the 1990 food-frequency questionnaire

<table>
<thead>
<tr>
<th></th>
<th>AHEI</th>
<th>DQI-R</th>
<th>RFS</th>
<th>aMED</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEI</td>
<td>0.60</td>
<td>0.80</td>
<td>0.69</td>
<td>0.60</td>
</tr>
<tr>
<td>AHEI</td>
<td>1.00</td>
<td>0.64</td>
<td>0.56</td>
<td>0.75</td>
</tr>
<tr>
<td>DQI-R</td>
<td>1.00</td>
<td>0.57</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>RFS</td>
<td>1.00</td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 n = 660. HEI, Healthy Eating Index; AHEI, Alternate Healthy Eating Index; DQI-R, Diet Quality Index Revised; RFS, Recommended Food Score; aMED, alternate Mediterranean Diet Index. All P values are < 0.0001.
<table>
<thead>
<tr>
<th></th>
<th>CRP (mg/L)</th>
<th>IL-6 (mg/L)</th>
<th>E-selectin (ng/L)</th>
<th>sICAM-1 (µg/L)</th>
<th>sVCAM-1 (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q1</td>
<td>Q3</td>
<td>Q5</td>
<td>Q1</td>
<td>Q3</td>
</tr>
<tr>
<td>HEI</td>
<td>2.0 ± 10.1</td>
<td>2.7 ± 2.8</td>
<td>1.7 ± 2.1</td>
<td>0.01</td>
<td>2.8 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>(36.0–66.4)</td>
<td>(74.6–80.5)</td>
<td>(86.6–98.7)</td>
<td></td>
<td>(36.0–66.4)</td>
</tr>
<tr>
<td>AHEI</td>
<td>3.8 ± 4.5</td>
<td>1.1 ± 13.5</td>
<td>2.1 ± 2.4</td>
<td>&lt;0.0001</td>
<td>3.5 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>(157–323)</td>
<td>(38.9–44.6)</td>
<td>(518–78.5)</td>
<td></td>
<td>(157–323)</td>
</tr>
<tr>
<td>DQIR</td>
<td>2.4 ± 10.4</td>
<td>2.2 ± 9.7</td>
<td>1.7 ± 9.0</td>
<td>0.005</td>
<td>2.7 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>(321–54.7)</td>
<td>(63.0–70.4)</td>
<td>(804–93.9)</td>
<td></td>
<td>(321–54.7)</td>
</tr>
<tr>
<td>RFS</td>
<td>2.0 ± 9.6</td>
<td>2.9 ± 4.0</td>
<td>1.6 ± 9.8</td>
<td>0.29</td>
<td>2.8 ± 3.3</td>
</tr>
<tr>
<td>aMED</td>
<td>3.4 ± 4.0</td>
<td>1.3 ± 13.4</td>
<td>2.5 ± 2.8</td>
<td>0.002</td>
<td>2.9 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>(0–2)</td>
<td>(4)</td>
<td>(5)</td>
<td></td>
<td>(0–2)</td>
</tr>
</tbody>
</table>
Correlations between the diet-quality scores were relatively high because many of the scores were based on similar dietary recommendations (Table 2). The highest correlation, \( r = 0.80 \) (\( P < 0.0001 \)), was between the HEI and DQI-R. The actual values of the biomarkers at the top, middle, and bottom quintiles of each diet-quality score are shown in Table 3. Differences in biomarker values between extreme quintiles of diet-quality scores were the greatest for the AHEI and aMED (Figure 1). We compared biomarker differences between the 5th and 1st quintiles for the different diet-quality scores. The only significant difference was between HEI and AHEI for sICAM-1 and sVCAM-1.

After age, BMI, smoking status, physical activity, and energy and alcohol intakes were adjusted for, we found that the HEI, DQI-R, and RFS were, in general, not significantly associated with biomarker concentrations (Table 4). However, each 1-z score increase on the AHEI and aMED was associated with significantly lower concentrations of many of these biomarkers. Of all the diet-quality scores, AHEI appeared to have the strongest associations with biomarker concentrations; the differences between the 5th and 1st quintiles of the AHEI scores ranged from 8% for concentrations of sICAM-1 and sVCAM-1 to 31% for concentrations of IL-6 (Figure 1). Higher aMED scores were also associated with a more favorable biomarker profile. Comparing top to bottom quintiles of aMED score, we found a reduction in biomarker concentrations of 24% in CRP, 16% in IL-6, and 13% in e-selectin concentrations. After additional adjustment for BMI, DQI-R scores were not associated with any biomarker concentrations (Table 4), and HEI and RFS scores were associated with a lower concentration of E-selectin only. Our results...
TABLE 4
β Coefficients for a 1-σ score increase in diet-quality scores

<table>
<thead>
<tr>
<th>Score</th>
<th>CRP (mg/L)</th>
<th>IL-6 (ng/L)</th>
<th>E-selectin (ng/L)</th>
<th>sICAM-1 (μg/L)</th>
<th>sVCAM-1 (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age and energy adjusted</td>
<td>−0.44&lt;sup&gt;2&lt;/sup&gt;</td>
<td>−0.33&lt;sup&gt;2&lt;/sup&gt;</td>
<td>−4.49&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−12.92&lt;sup&gt;2&lt;/sup&gt;</td>
<td>−8.08</td>
</tr>
<tr>
<td>MV adjusted</td>
<td>−0.32&lt;sup&gt;3&lt;/sup&gt;</td>
<td>−0.22</td>
<td>−3.03&lt;sup&gt;1&lt;/sup&gt;</td>
<td>−5.50</td>
<td>−5.35</td>
</tr>
<tr>
<td>MV + BMI adjusted</td>
<td>−0.04</td>
<td>−0.20</td>
<td>−1.93</td>
<td>−3.86</td>
<td>−2.17</td>
</tr>
<tr>
<td>AHEI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age and energy adjusted</td>
<td>−0.65&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−0.40&lt;sup&gt;2&lt;/sup&gt;</td>
<td>−6.31&lt;sup&gt;1&lt;/sup&gt;</td>
<td>−14.47&lt;sup&gt;2&lt;/sup&gt;</td>
<td>−14.39&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>MV adjusted</td>
<td>−0.60&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−0.33&lt;sup&gt;3&lt;/sup&gt;</td>
<td>−5.57&lt;sup&gt;1&lt;/sup&gt;</td>
<td>−8.15&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−13.85</td>
</tr>
<tr>
<td>MV + BMI adjusted</td>
<td>−0.36&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−0.30&lt;sup&gt;3&lt;/sup&gt;</td>
<td>−4.74&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−6.93&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−11.04</td>
</tr>
<tr>
<td>DQI-R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age and energy adjusted</td>
<td>−0.48&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−0.24&lt;sup&gt;3&lt;/sup&gt;</td>
<td>−4.26&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−9.73&lt;sup&gt;2&lt;/sup&gt;</td>
<td>−11.42</td>
</tr>
<tr>
<td>MV adjusted</td>
<td>−0.38&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−0.14</td>
<td>−2.94&lt;sup&gt;3&lt;/sup&gt;</td>
<td>−2.83</td>
<td>−10.15</td>
</tr>
<tr>
<td>MV + BMI adjusted</td>
<td>−0.07</td>
<td>−0.11</td>
<td>−1.70</td>
<td>−1.30</td>
<td>−6.82</td>
</tr>
<tr>
<td>RFS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age and energy adjusted</td>
<td>−0.22</td>
<td>−0.17</td>
<td>−3.70&lt;sup&gt;3&lt;/sup&gt;</td>
<td>−5.51&lt;sup&gt;2&lt;/sup&gt;</td>
<td>−7.39</td>
</tr>
<tr>
<td>MV adjusted</td>
<td>−0.10</td>
<td>−0.08</td>
<td>−2.39&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−2.53</td>
<td>−5.12</td>
</tr>
<tr>
<td>MV + BMI adjusted</td>
<td>0.05</td>
<td>−0.11</td>
<td>−2.38&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−2.25</td>
<td>−3.57</td>
</tr>
<tr>
<td>aMED</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age and energy adjusted</td>
<td>−0.57&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−0.37&lt;sup&gt;3&lt;/sup&gt;</td>
<td>−4.63&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−11.35&lt;sup&gt;2&lt;/sup&gt;</td>
<td>−15.57&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>MV adjusted</td>
<td>−0.50&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−0.31&lt;sup&gt;2&lt;/sup&gt;</td>
<td>−3.90&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−7.38&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−14.99&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>MV + BMI adjusted</td>
<td>−0.36&lt;sup&gt;2&lt;/sup&gt;</td>
<td>−0.29&lt;sup&gt;2&lt;/sup&gt;</td>
<td>−3.48&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−6.84</td>
<td>−13.07&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> n = 660. MV, multivariate linear regression adjusted for age, alcohol intake, smoking status, physical activity, and total energy intake; HEI, Healthy Eating Index; AHEI, Alternate Healthy Eating Index; DQI-R, Diet Quality Index Revised; RFS, Recommended Food Score; aMED, alternate Mediterranean Diet Index; sICAM-1, soluble intercellular cell adhesion molecule 1; sVCAM-1, soluble vascular cell adhesion molecule 1; CRP, C-reactive protein; IL-6, interleukin 6.<n> <sup>2</sup> P < 0.01.<n> <sup>3</sup> P < 0.0001.<n> <sup>4</sup> P < 0.05.

remained essentially the same when alcohol intake was not included in the regression models.

DISCUSSION

We compared the associations between the different diet-quality scores and biomarkers of inflammation and endothelial dysfunction. We found that the AHEI and aMED scores had the strongest inverse associations with these biomarkers and that the HEI, DQI-R, and RFS scores had little association with these biomarkers.

Differences in associations between indexes and biomarkers of inflammation and endothelial dysfunction are likely attributable to differences in the food and nutrient components of each index. For example, both the AHEI and aMED scores focus on dietary patterns high in fruit and vegetables, whole grains, nuts, and fish and moderate in alcohol, and these 2 scores were highly correlated in our sample. The AHEI further awards points to diets with a high ratio of polyunsaturated to saturated fat, whereas the aMed score also awards points for diets with a high ratio of monounsaturated to saturated fat. Therefore, these 2 scoring systems are unique in that they can capture diets high in long-chain n–3 fatty acids. Previous studies have shown an association between the intake of long-chain n–3 fatty acids and lower concentrations of inflammatory markers (7). Recent studies have continued to show favorable changes in CRP, IL-6, and sVCAM-1 with supplementation with α-linolenic acid or oils rich in long-chain n–3 fatty acids (26–28). On the other hand, diets high in trans fat or saturated fat were found to be associated with higher concentrations of CRP, IL-6, and E-selection in a 5-wk randomized crossover study in men (29). In another randomized trial, a diet low in saturated fat and high-fat dairy products but high in refined grains was effective at lowering CRP concentrations but was not as effective as were lovastatin and a diet high in plant sterols, soy protein, viscous fiber, and almonds (30).

The lack of association of RFS, DQI-R, and HEI with biomarkers of inflammation and endothelial dysfunction may be explained by the nonspecificity of fat and carbohydrate quality, emphasis on lower total fat intake, and broad inclusion of many foods in these indexes. The HEI is based on the food guide pyramid, which gives more points for a diet low in all types of fat, including unsaturated fats. It also allows individuals to score higher points if their diet contains refined grains. The scoring criteria also included sodium intake and diet variety. Although meaningful for an overall healthy diet, these components are not specific to inflammation. The DQI-R is similar to the HEI, and these 2 scores were highly correlated in the present study. However, the DQI-R also includes calcium and iron intakes, which again, may not be related to inflammation. The RFS criteria include intakes of vegetables, fruit, healthy protein sources, grains, and dairy products but it also does not distinguish between different types of fatty acids or penalize for consumption of items that are not recommended. The AHEI but not the HEI and RFS, has been shown to predict cardiovascular disease risk in women (14, 19). High scores on the HEI, DQI-R, and RFS do not capture differences in types of fats, which probably contributed to the poor performance of these indexes in the present study. Therefore, although these indexes may reflect certain aspects of diet quality, they may be poor choices for evaluating diet quality.
specifically for reducing the risk of diseases influenced by inflammation, such as cardiovascular disease and diabetes.

In our analysis, the HEI and DQI-R scores were somewhat higher than those obtained in the US Continuing Survey of Food Intakes by Individuals 1994 –1996 (12, 19): 77 compared with 64 and 68 compared with 63, respectively. However, in our sample, the mean score of the 1st quintile was 59 with both the HEI and the DQI-R, and the mean score of the 5th quintile was 91 with the HEI and 86 with the DQI-R. Therefore, we still had a substantial contrast in the distribution of diet-quality scores in our sample to detect any association with biomarkers.

In our analysis, the AHEI was the strongest correlate for IL-6, which decreased by 0.30 ng/L for each 1–z score increase on the AHEI (Table 4). This finding is comparable with the difference between obese and normal-weight (3.18 compared with 1.4 pg/mL) premenopausal women and in obese women after a weight loss of ≥10% (31). In a comparison of the top- and bottom-quintile AHEI scores, the reduction in sICAM-1 and sVCAM-1 in our sample (8%) was similar to that for obese women who sustained a weight loss of ≥10% (31). Because we adjusted our analysis for BMI and only studied women free of disease, our study results suggest that the AHEI is capable of capturing associations between diet and markers of inflammation even in healthy-weight individuals, independent of adiposity.

This analysis was controlled extensively for potential lifestyle predictors of biomarkers; therefore, any confounding would be minimal. However, because of the cross-sectional nature of this study, we cannot infer causality from our results. Diet-quality indexes comprise a combination of many food groups or food components. Components of each of the indexes could have different influences on biomarkers. In addition, diet-quality indexes or their components may influence more than one pathway of endothelial dysfunction and inflammation. The diet-quality indexes that we examined were based on North American dietary habits. It is important to examine dietary patterns in other populations (eg, Asian and Mediterranean) and their relations with biomarkers for the development of chronic diseases.

In conclusion, we found that dietary indexes that reflect current intake guidelines were not predictive of biomarkers of inflammation and endothelial dysfunction. In contrast, the AHEI and aMED scores were strongly associated with lower concentrations of biomarkers of inflammation and endothelial dysfunction. Because these pathologic processes are linked to the development of cardiovascular disease and diabetes, our data suggest a possible mechanism for the role of diet quality in relation to the risk of diabetes and cardiovascular disease. Therefore, these 2 diet-quality indexes may be useful as guidelines for reducing the risk of diseases.

TTF designed and conducted the analysis and drafted the manuscript. PKN and MLM assisted with the statistical analysis. JEM, WCW, FBH, and TTF secured funding for the project. NR analyzed the blood samples. All authors participated in the revision and approval of the manuscript. None of the authors had a conflict of interest.

REFERENCES

**APPENDIX A**

Scoring criteria for diet-quality indexes

<table>
<thead>
<tr>
<th>Component</th>
<th>Foods included</th>
<th>≤50 y</th>
<th>≥51 y</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grains</td>
<td>Cooked and cold cereals, loaf breads and quick breads, rice, pasta, pizza, pancakes, pies, crackers, cookies, other grains, brownies, donuts, cakes, sweet rolls, bran, wheat germ, chowder</td>
<td>9.1 servings/d</td>
<td>7.4 servings/d</td>
<td>10; 1 point less for each 10% less than intake required for full score</td>
</tr>
<tr>
<td>Vegetables</td>
<td>All vegetables, potato products, pizza, chowder, tomato products</td>
<td>4.2 servings/d</td>
<td>3.5 servings/d</td>
<td>Same as above</td>
</tr>
<tr>
<td>Fruit</td>
<td>Fruits, fruit juices, pies</td>
<td>3.2 servings/d</td>
<td>2.5 servings/d</td>
<td>Same as above</td>
</tr>
<tr>
<td>Milk</td>
<td>Milk, sherbet, ice cream, yogurt, cheeses, pizza, potatoes, chocolate, chocolate candies, chowder</td>
<td>2.0 servings/d</td>
<td>2.0 servings/d</td>
<td>Same as above</td>
</tr>
<tr>
<td>Meat</td>
<td>Eggs, chicken, processed meats, red meats, seafood, tofu, soymilk, tree nuts, peanuts, peanut butter, chocolate, brownie, organ meats, beans, chowder</td>
<td>2.4 servings/d</td>
<td>2.2 servings/d</td>
<td>Same as above</td>
</tr>
<tr>
<td>Total fat</td>
<td>≤30% of energy</td>
<td>≤30% of energy</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31–44 of energy</td>
<td>31–44 of energy</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥45 of energy</td>
<td>≥45 of energy</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Saturated fat</td>
<td>≤10% of energy</td>
<td>≤10% of energy</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11–14% of energy</td>
<td>11–14% of energy</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥15% of energy</td>
<td>≥15% of energy</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>&lt;300 mg</td>
<td>&lt;300 mg</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>301–449 mg</td>
<td>301–449 mg</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥450 mg</td>
<td>≥450 mg</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Sodium</td>
<td>≤2400 mg</td>
<td>≤2400 mg</td>
<td>10; 1 point less for each 10% less than intake required for full score</td>
<td></td>
</tr>
<tr>
<td>Variety</td>
<td>Top 10% intake of the sum of unique foods</td>
<td>Top 10% intake of the sum of unique foods</td>
<td>Same as above</td>
<td></td>
</tr>
</tbody>
</table>

1 The amount of food in each food group (e.g., the amount of milk in chowder) was adjusted according to a recipe so that food with small amounts of the food group would not contribute excessively to that food group.
2 Based on 2200 kcal for the ≤50 y category and 1900 kcal for the ≥51 y category.
<table>
<thead>
<tr>
<th>Component</th>
<th>Foods included</th>
<th>Criteria</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetables</td>
<td>All vegetables, tomato products, yams, pizza (does not include potatoes)</td>
<td>5 servings/d</td>
<td>10; 1 point less for each 10% less than intake required for full score</td>
</tr>
<tr>
<td>Fruit</td>
<td>All fruit and fruit juices</td>
<td>4 servings/d</td>
<td>Same as above</td>
</tr>
<tr>
<td>Nuts and soy</td>
<td>Tree nuts, peanuts, peanut butter, tofu, soymilk</td>
<td>1 servings/d</td>
<td>Same as above</td>
</tr>
<tr>
<td>Ratio of white to red meat</td>
<td>White: chicken, seafood; red: processed meats, red meats, organ meats</td>
<td>4</td>
<td>Same as above</td>
</tr>
<tr>
<td>Cereal fiber</td>
<td>—</td>
<td>15 g/d</td>
<td>Same as above</td>
</tr>
<tr>
<td>trans Fat</td>
<td>—</td>
<td>≤0.5% of energy</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>&gt;0.5 but &lt;4.0% of energy</td>
<td>1 point less for each 10% increment in this range</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>≥4</td>
<td>0</td>
</tr>
<tr>
<td>Ratio of polyunsaturated to saturated fat</td>
<td>—</td>
<td>≥1</td>
<td>10; 1 point less for each 10% less than intake required for full score</td>
</tr>
<tr>
<td>Long-term multivitamin use</td>
<td>—</td>
<td>≥5 y</td>
<td>7.5 points for ≥5 y regular use; 2.5 for all others</td>
</tr>
<tr>
<td>Alcohol</td>
<td>Red and white wine, beer, “light” beer, liquor</td>
<td>Men: 1.5–2.5 servings/d; women: 0.5–1.5 servings/d</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intake &lt; “ideal”</td>
<td>1 point less for each 10% less than ideal intake</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intake &gt; “ideal”</td>
<td>1 point less for each 10% above than ideal intake</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Men: 0 or &gt; 3.5 servings/d; women: 0 or &gt;2.5 servings/d</td>
<td>0</td>
</tr>
</tbody>
</table>
### Diet Quality Index Revised (3)

<table>
<thead>
<tr>
<th>Component</th>
<th>Foods included</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Grains</strong></td>
<td>Crackers, cereals, pizza, dark breads, rice, bran, wheat germ, breads, oatmeal,</td>
<td>≥9 servings/d 10; 1 point less for each 10% less than intake required for full score</td>
</tr>
<tr>
<td></td>
<td>pasta, English muffins, muffins, pancakes, other grains</td>
<td>≥7.4 servings/d 51 y</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Same as above</td>
</tr>
<tr>
<td><strong>Vegetables</strong></td>
<td>All vegetables, yams, potatoes, French fries, corn, tomato juice, tomato sauce,</td>
<td>≥4 servings/d 10; 1 point less for each 10% less than intake required for full score</td>
</tr>
<tr>
<td></td>
<td>tofu, legumes, tofu</td>
<td>≥3.5 servings/d Same as above</td>
</tr>
<tr>
<td><strong>Fruit</strong></td>
<td>All fruit and juices (except tomato juice)</td>
<td>≥3 servings/d 10; 1 point less for each 10% less than intake required for full score</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥2.5 servings/d Same as above</td>
</tr>
<tr>
<td><strong>Total fat</strong></td>
<td>≤30% of energy</td>
<td>Same as for &lt;50 y 10</td>
</tr>
<tr>
<td></td>
<td>30.1–40% of energy</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>&gt;40% of energy</td>
<td>0</td>
</tr>
<tr>
<td><strong>Saturated fat</strong></td>
<td>≤10% of energy</td>
<td>Same as for &lt;50 y 10</td>
</tr>
<tr>
<td></td>
<td>10.1–13% of energy</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>&gt;13% of energy</td>
<td>0</td>
</tr>
<tr>
<td><strong>Cholesterol</strong></td>
<td>≤300 mg</td>
<td>Same as for &lt;50 y 10</td>
</tr>
<tr>
<td></td>
<td>301–400 mg</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>&gt;400 mg</td>
<td>0</td>
</tr>
<tr>
<td><strong>Calcium (AI for age)</strong></td>
<td>1000 mg 1200 mg</td>
<td>Maximum of 2.5 points for each category; maximum of 10; total diversity = grains + vegetables + fruit + protein</td>
</tr>
<tr>
<td><strong>Iron (RDA for age)</strong></td>
<td>18 mg 8 mg</td>
<td>Same as above</td>
</tr>
<tr>
<td><strong>Diet diversity (based on sum of 4 categories)</strong></td>
<td>Grains: non-whole-grain breads, quick breads, whole-grain breads, pasta, cereals, rice, other grains</td>
<td>≥0.25 servings/d of each food = 1 point; the total for each category is averaged by the number of foods in the category (eg, 3 for fruit) and then multiplied by 2.5</td>
</tr>
<tr>
<td></td>
<td>Vegetables: yellow and orange, deep green (spinach, broccoli); tomato products,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>beans, tofu, soy, starch vegetables, other vegetables</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Fruit</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>citrus, berry, melon, juices, other fruit</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Protein</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>beef, pork, organ meat, deli meat, chicken, milk, cheese, eggs, soups, seafood,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>yogurt</td>
<td></td>
</tr>
<tr>
<td><strong>Diet moderation</strong></td>
<td>Amount of added fat from cream, butter, margarine, cream cheese, oil and vinegar</td>
<td>Added fat: 2.5 g/d 2.5</td>
</tr>
<tr>
<td></td>
<td>dressing, chocolate, whole milk, sour cream, ice cream, mayonnaise and creamy</td>
<td>25.1–50 g 1.0</td>
</tr>
<tr>
<td></td>
<td>dressings, other cheese, cookies, chowder and cream soups, sherbet, French fries,</td>
<td>≥50.1–75 g 0</td>
</tr>
<tr>
<td></td>
<td>brownies, muffins and biscuits, donuts, pancakes and waffles, cakes, pies, sweet</td>
<td>&gt;75 g 0</td>
</tr>
<tr>
<td></td>
<td>rolls, coffee cake, pastries</td>
<td></td>
</tr>
<tr>
<td><strong>Sodium</strong></td>
<td>≤2400 mg</td>
<td>Same as for &lt;50 y 2.5</td>
</tr>
<tr>
<td></td>
<td>2401–3400 mg</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>&gt;3400 mg</td>
<td>0</td>
</tr>
<tr>
<td><strong>Alcohol (women):</strong></td>
<td>≤1 drink/d</td>
<td>Same as for &lt;50 y 2.5</td>
</tr>
<tr>
<td></td>
<td>1.01–1.5 drink/d</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>≥1.51 drink/d</td>
<td>0</td>
</tr>
<tr>
<td>**Teaspoons of added sugar from sweeteners, sodas, candy bars, candy bars,</td>
<td>Added sugar: 2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cookies, cakes, sweet rolls, coffee cake, pastries, jam honey, sherbet, ice cream</td>
<td>≤7.5 tsp/d 2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.6–11.25 tsp/d 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.26–15 tsp/d 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;15 tsp/d 0</td>
</tr>
</tbody>
</table>

Total score = sodium + alcohol + added fat + added sugar

---

1. AI: adequate intake; RDA: recommended dietary allowance.
2. Based on 2200 kcal for the <50 y category and 1900 kcal for the >51 y category.
### Recommended Food Score (2, 4)

<table>
<thead>
<tr>
<th>Food group</th>
<th>Foods included</th>
<th>Maximum score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetables</td>
<td>Tomatoes, broccoli, spinach, kale, carrots, iceberg lettuce, yams, potatoes, beans, string beans, corn, peas, mixed vegetables, celery, yellow squash, eggplant, romaine lettuce, tomato juice, tomato sauce, cabbage, cauliflower, Brussels sprouts, beets</td>
<td>51</td>
</tr>
<tr>
<td>Fruit</td>
<td>Apples or pears, oranges, cantaloupe, orange juice, grapefruit juice, grapefruit, other fruit juices, banana, apple juice, strawberries, blueberries, peaches, raisins, watermelon, applesauce, prunes</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Chicken or turkey without skin, other fish, dark fish, canned tuna, tofu, shrimp</td>
<td></td>
</tr>
<tr>
<td>Grains</td>
<td>Dark breads, whole-grain cereals (predefined write-ins), cooked cereals, oatmeal, brown rice</td>
<td></td>
</tr>
<tr>
<td>Dairy</td>
<td>Skim milk</td>
<td></td>
</tr>
</tbody>
</table>

1 point for each item consumed at least weekly.

### Alternate Mediterranean Diet Score (5, 6)

<table>
<thead>
<tr>
<th>Food group</th>
<th>Foods included</th>
<th>Criteria for 1 point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetables</td>
<td>All vegetables except potatoes</td>
<td>Greater than median intake (servings/d)</td>
</tr>
<tr>
<td>Legumes</td>
<td>Tofu, string beans, peas, beans</td>
<td>Greater than median intake (servings/d)</td>
</tr>
<tr>
<td>Fruit</td>
<td>All fruit and juices</td>
<td>Greater than median intake (servings/d)</td>
</tr>
<tr>
<td>Nuts</td>
<td>Nuts, peanut butter</td>
<td>Greater than median intake (servings/d)</td>
</tr>
<tr>
<td>Whole grains</td>
<td>Whole-grain ready-to-eat cereals, cooked cereals, crackers, dark breads, brown rice, other grains, wheat germ, bran, popcorn</td>
<td>Greater than median intake (servings/d)</td>
</tr>
<tr>
<td>Red and processed meats</td>
<td>Hot dogs, deli meat, bacon, hamburger, beef</td>
<td>Less than median intake (servings/d)</td>
</tr>
<tr>
<td>Fish</td>
<td>Fish and shrimp, breaded fish</td>
<td>Greater than median intake (servings/d)</td>
</tr>
<tr>
<td>Ratio of monounsaturated to saturated fat</td>
<td>—</td>
<td>Greater than median intake (servings/d)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Wine, beer, “light” beer, liquor</td>
<td>5–25 g/d</td>
</tr>
</tbody>
</table>

0 points if these criteria are not met.

### REFERENCES

Compliance with expert population-based dietary guidelines and lower odds of carotid atherosclerosis in women: the Framingham Nutrition Studies1–3

Barbara E Millen, Paula A Quatromoni, Byung-Ho Nam, Michael J Pencina, Joseph F Polak, Ruth W Kimokoti, Jose M Ordovas, and Ralph B D’Agostino

ABSTRACT
Background: Carotid stenosis, an indicator of subclinical atherosclerosis, predicts future coronary artery disease (CAD) and stroke and provides a noninvasive method to identify candidates for primary prevention. The relation between diet and stenosis is relatively unexplored, particularly in women.
Objective: We evaluated in women the association between nutrient intakes that were consistent with expert population-based dietary guidelines and carotid stenosis.
Design: We used prospective logistic regression analyses to evaluate relations between baseline nutrient intake and the presence of carotid stenosis at 4-y follow-up in 1123 women from the Framingham Offspring-Spouse study, after control for multiple CAD risk factors. We also developed multivariate models that were stratified by compliance with expert population-based dietary guidelines and smoking status.
Results: Baseline nutrient and risk factor profiles differed by women’s compliance and smoking status. Dietary noncompliance and smoking were each associated with odds for stenosis that were 2.5-fold those of dietary compliance and nonsmoking. Odds were highest for dietary noncompliance in combination with smoking (odds ratio: 3.49; 95% CI: 1.67, 7.27).
Conclusions: Nutrient intake consistent with current expert population-based dietary guidelines and smoking abstinence are associated with lower odds of carotid atherosclerosis in women. Unique dietary and risk factor profiles of at-risk women suggest areas for targeted primary CAD prevention.

KEY WORDS Carotid stenosis, total and saturated fat, dietary cholesterol, smoking, preventive nutrition, coronary artery disease, CAD

INTRODUCTION
Coronary artery disease (CAD) remains the leading cause of morbidity and mortality in adult Americans; it accounts for nearly 500,000 deaths annually and contributes to the $300 billion in health care costs and lost individual productivity that is associated with cardiovascular disease (CVD) (1, 2). The primary prevention of CAD, particularly among men and women who have multiple CVD risk factors, is a national public health priority (3–7).

Healthy People 2010 focuses on preventing CAD in addition to other chronic diseases (eg, stroke, obesity, diabetes, and cancer) and on promoting health by encouraging good nutrition and physical activity and the avoidance of smoking (4). The current US Department of Agriculture (USDA) Dietary Guidelines for Americans 2000 (5) and the AHA [American Heart Association] Dietary Guidelines (6), which include lifestyle-related recommendations for CAD prevention, emphasize the population benefits of achieving and maintaining an overall healthy eating pattern, appropriate body weight, desirable lipid and blood pressure measurements, daily physical activity, and the avoidance of smoking. Specific population-based nutrient guidelines to lower CAD risk factor profiles recommend the following daily intakes: ≤30% of total energy as fat, <10% of energy as saturated fat, and <300 mg cholesterol (5, 6). Although these guidelines are similarly applicable to men and women, an even greater potential may exist for primary prevention in women because of the 10–15-y delay in the onset of CAD in women compared with men (7).

Over the past decade, increases in physician and public awareness of the benefits of reducing cholesterol concentrations, improvements in American dietary lipid and cholesterol intakes, and lower population serum cholesterol concentrations have contributed to declining rates of CAD. Nonetheless, recent reports (5, 7) emphasize that future success in reducing the national

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2 Supported by grants no. R01-HL-60700 and N01-HC-38038 from the National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD.
3 Address reprint requests to B Millen, Department of Social and Behavioral Sciences, Room 263W, Boston University School of Public Health, 715 Albany Street, Talbot 2 West, Boston, MA 02118. E-mail: bmillen@bu.edu.
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burden of heart disease will depend on identifying effective prevention strategies, particularly those that focus on the following: primary prevention of CAD in the population, feasible targets for behavioral lifestyle modifications, and effective methods to promote long-term behavior change. Experts and clinicians also seek continuing evidence that the existing population-based lifestyle-related guidelines for CAD prevention promote CAD risk reduction (5, 7), including lowering the risk of preclinical CAD. Such research is particularly important in light of the dramatic rise in the proportion of American adults who are overweight and obese and the low rates of leisure-time physical activity noted in the US population (8, 9). In this study, we evaluated in women the relation between compliance with the USDA and AHA dietary guidelines and carotid artery stenosis, an indicator of subclinical atherosclerosis. Carotid stenosis predicts future fatal and nonfatal CAD and stroke (10–17) and offers a noninvasive method of identifying candidates for primary CAD-prevention activities (18, 19).

SUBJECTS AND METHODS

Subjects

The Framingham Study began in 1948 as a longitudinal, population-based study of CVD among residents of Framingham, MA (20; also RB D’Agostino, WB Kannel, unpublished observations, 1988–1989). In 1971, a second-generation cohort was recruited when some 5124 Framingham Study offspring and their spouses were invited to participate in the Framingham Offspring-Spouse (FOS) study (21).

Members of the FOS cohort are examined in the Framingham Study clinic, on average, every 4 y. They participate in a standardized protocol that involves a complete physical examination, laboratory tests, noninvasive diagnostic testing, and updating of medical histories and other pertinent information. At certain examinations, detailed dietary data are collected. The dietary and risk factor data reported here were collected among FOS women at Exam 5, between 1992 and 1996. Some 2007 women, aged 26–84 y, participated in this examination (76% of the original FOS cohort women). Women who were diagnosed with CVD at or before Exam 5 were excluded from these analyses (6.7%; n = 135).

All participants provided written informed consent. This study was approved by the institutional review board at Boston University Medical Center.

Nutrient intake and dietary compliance

Nutrient intake was estimated from 3-d dietary records collected with the use of a standardized, published method (22, 23). Participants were instructed by a registered dietitian in the clinic to record their intake over 2 weekdays and 1 weekend day, while adhering to their usual eating practices. Subjects were trained to estimate portion sizes with the use of a validated two-dimensional food portion visual aid (23). Some 68.5% of women (n = 1375) completed the dietary record protocol. Dietary records were processed by trained coders who adhered to standardized protocols. Nutrient calculations were performed with the use of the MINNESOTA NUTRITION DATA SYSTEM software (version 2.6; Food Database 6A; Nutrient Database 23; Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN; 24).

Subjects who were considered compliant had a 3-d dietary mean nutrient intake that met the population-based dietary guidelines for CAD risk reduction as set forth in the AHA Dietary Guidelines and that was consistent with the USDA’s Dietary Guidelines for Americans, including a total of ≤30% of energy as fat (kcal), <10% of energy as saturated fat (kcal), and <300 mg dietary cholesterol (4–6). Noncompliant subjects consumed diets that failed to meet ≥1 of these guidelines. We compared these dietary guidelines with the medical nutrition therapy recommendations (7), to assess the potential benefits associated with population-based recommendations in relation to an early indicator of atherosclerotic heart disease.

Assessment of carotid atherosclerosis

Among 1283 women who provided complete dietary records and who were free of CVD at baseline, the presence of carotid atherosclerosis was assessed at follow-up at Exam 6 (1996–1999). Carotid ultrasound scanning studies were obtained for 1137 subjects (89% of the total cohort). Missing measurements were exclusively due to logistic constraints at the clinic (eg, unavailability of the ultrasound scanning device or the sonographer during the scheduled visit). Reliability studies were ongoing during data acquisition.

Ultrasound imaging studies were conducted with a high-resolution linear-array 5.0 MHz transducer and color Doppler ultrasound scanning device (Toshiba SSH-140; Toshiba Medical Systems, Tustin, CA). Imaging was performed with the subject’s head rotated 45 degrees away from the side being studied, according to a standard protocol. Two gray-scale images were taken at the level of the common carotid artery bulb, and 2 additional images were obtained in the proximal 2 cm of the internal carotid artery. One image of the respective image pairs was acquired with the probe held at 45 degrees from horizontal. For the second image, the sonographer was instructed to position the transducer to best identify any focal lesions. All images were gated to the R-wave of an electrocardiogram, and both sides of the neck were imaged. Images were directly transferred into a computer workstation through a frame-grabber board.

Color Doppler imaging and pulsed Doppler waveforms were used to evaluate blood flow velocities in the proximal internal carotid arteries. Angle-corrected Doppler velocity waveforms were acquired in the proximal internal carotid artery at the site of highest velocity as identified on a color Doppler image. Peak systolic velocities were measured from these tracings. A certified reader reviewed the acquired digital images and made a subjective estimate of the degree of internal carotid artery narrowing, which was graded as 0%, 1–24%, or 25–49% when Doppler-derived peak systolic velocities in the internal carotid artery were <150 cm/s. Internal carotid artery disease was characterized by the maximum stenosis observed on the right or left side and was categorized as 0% (no lesions), 1–24%, 25–49% (focal lesions causing <50% diameter stenosis), or ≥50% (lesions causing ≥50% diameter stenosis). Absence of blood flow corresponded to a total occlusion.

CVD risk factor measurements

CVD risk factors are routinely measured at all Framingham Study examinations (25). All lipid analyses were performed at the Framingham Study laboratory, which participates in the Standardization Program of the Centers for Disease Control and
Prevention and the National Heart, Lung, and Blood Institute Lipid Research Clinics. Venous blood was drawn from all subjects after a 12–24-h fast. Total and HDL-cholesterol concentrations were measured by automated enzymatic methods (26, 27). The cholesterol content of LDL cholesterol was estimated by the method of Friedewald et al (28). Triacylglycerol concentrations were measured enzymatically (26). Blood pressure was determined by duplicate measurements of the subject’s left arm made with the use of a mercury sphygmomanometer while the subject was in a sitting position. Body mass index (BMI; in kg/m²) was calculated from height and weight values measured in the clinic. Diabetes was defined as a history of use of insulin or an oral hypoglycemic agent or a fasting blood glucose concentration of ≥7.8 mmol/L (≥140 mg/dL).

Physical activity was measured with the use of a standardized questionnaire to determine estimates of activity in a usual day, based on a 24-h history. A physical activity index was calculated from the number of hours spent doing specific activities that were categorized (ie, sedentary, slight, moderate, or heavy) and weighted according to the oxygen consumption required to perform them (29). Physical activity index scores ranged from 24 (total bed rest) to 120. Because physical activity was not measured at FOS Exam 3, Exam 2 values were substituted in these analyses, according to the analytic approach routinely used in the Framingham study. Menopausal status and cigarette smoking was self-reported. Pack-years of cigarette smoking were defined as the number of packs of cigarettes smoked per day multiplied by the total number of years a person smoked. This variable takes into account the duration of smoking as well as the amount of cigarette consumption.

Statistical analysis

Our primary objective was to determine whether compliance with the current population-based dietary guidelines for CAD risk reduction (as defined earlier) was related to the presence of carotid atherosclerosis at follow-up. The endpoint of interest was carotid artery stenosis, defined by focal lesions of ≥25% in either the right or left internal carotid artery, in accordance with our previously used threshold (30–32). Previous research suggested potential interaction between diet and smoking (32, 33); thus, we decided to test for such an interaction. Because the null hypothesis (ie, no interaction) could not be rejected ($P = 0.10$), we decided to stratify by compliance and smoking status and created 4 subgroups: compliant nonsmokers, noncompliant nonsmokers, compliant smokers, and noncompliant smokers.

For descriptive purposes, age-adjusted mean baseline Exam 5 CVD risk factors and nutrient intake profiles were computed for the 4 subgroups. Analysis of covariance was used for calculating the least-squares means of continuous variables by using PROC GLM in SAS (34). For categorical variables, age-adjusted proportions were computed by using the age-adjusted logistic regression (PROC LOGISTIC) (34). If the interaction between smoking and compliance was significant, we compared the 4 subgroups by using Bonferroni’s correction for multiple testing (34). In multivariate analyses, we examined the relation between dietary compliance and carotid atherosclerosis at follow-up with dietary compliance and smoking subgroups by using compliant nonsmokers as the reference group. Odds ratios (ORs) were calculated by using logistic regression in which CVD risk factors were considered separately in age- and energy-adjusted models. Models considered a range of CVD risk factors, including age, systolic and diastolic blood pressures, total cholesterol, LDL cholesterol, the ratio of total to HDL cholesterol, plasma triacylglycerols, BMI, physical activity level, menopausal status, and conditions that included obesity, diabetes, and hypertension. In addition, education level (high school or below compared with more than high school) was considered as a proxy indicator of socioeconomic status. The final multivariate model reported here is limited to those variables that were identified as important predictors or potential confounders of the relation of interest. The sample used in the analyses consisted of women with no missing values for carotid stenosis, dietary compliance, smoking, and the covariates retained in the final multivariate model ($n = 1123$). Analyses were carried out by use of SAS software (version 8.2; SAS Institute Inc, Cary, NC).

RESULTS

Among 1123 women studied, 360 (32%, both smokers and nonsmokers) had baseline age-adjusted mean daily nutrient intakes that complied with all 3 criteria of the USDA and AHA dietary guidelines for CAD risk reduction (5, 6): a total of ≤30% of energy as fat, <10% of energy as saturated fat, and <300 mg dietary cholesterol (Table 1). Some 68% of FOS women ($n = 763$) had nutrient profiles that failed to meet ≥1 of these current population dietary recommendations (noncompliant smokers and nonsmokers). Compliant and noncompliant subjects (both smokers and nonsmokers) also had markedly different intakes of a wider range of nutrients and CVD risk factors. Specifically, the diets of noncompliant women were higher in total energy, dietary lipids, cholesterol, and sodium; less concentrated in carbohydrate and fiber; and less nutrient-dense overall than were the diets of women who were compliant. Smokers (both compliant and noncompliant) had higher alcohol intakes than did nonsmokers. Noncompliant smokers had the lowest intakes of folate.

The baseline CVD characteristics of FOS women are presented in Table 2. Noncompliant women were younger and had lower total:HDL cholesterol than did compliant women. Compared with compliant smokers, noncompliant smokers had higher levels of cigarette exposure at baseline. The means of systolic and diastolic blood pressure; BMI; total, HDL, and LDL cholesterol; physical activity; and the rates of diabetes did not differ significantly between compliant and noncompliant subjects.

The multivariate models, stratified by the combination of dietary compliance and smoking exposure, are presented in Table 3. Compliant women who never smoked were the reference group. Compared with nonsmoking subjects who complied with the current dietary guidelines, noncompliant women who never smoked had 2.5-fold higher odds of carotid stenosis. The odds of carotid stenosis in women with compliant diets at baseline but who smoked was similar to the odds in noncompliant women who smoked. Noncompliant women who also smoked at baseline had odds of carotid atherosclerosis at 4 y of follow-up 3.5 times those in the reference group.

DISCUSSION

This is the first cohort study in women to examine the relation between compliance with population-based dietary guidelines for CAD prevention and the incidence of carotid stenosis, a subclinical marker of systemic atherosclerosis (10, 11, 35, 36)
That predicts future CAD and cerebrovascular events (10, 12–17, 37, 38). More than two-thirds of the women in the FOS Study had nutrient intakes that failed to meet the current population-based nutritional guidelines (from USDA and AHA) at baseline in 1992–1996. Dietary noncompliance and cigarette smoking each more than doubled the odds for stenosis seen with dietary compliance. Noncompliant women who smoked experienced a tripling of the odds of carotid stenosis.

These findings are consistent with emerging evidence on lifestyle behaviors in relation to carotid stenosis, CAD risk, and other health outcomes in adult populations. We have reported on the associations between the 5 distinct dietary patterns of FOS Study women and the presence of carotid stenosis at 12 y of follow-up (32). After multivariate adjustment for age, systolic blood pressure, BMI, total: HDL cholesterol, and pack-years of smoking, women whose dietary patterns were characterized as “empty calorie” (ie, more concentrated in high-fat, high-sugar foods and lower in fruit and vegetables and micronutrient-rich foods) were compared with women with more heart-healthy diets (ie, higher intakes of vegetables, fruit, and low-fat foods), and it was found that the former group had a significantly higher risk of carotid stenosis at 12-y follow-up [relative risk (RR): 2.28; 95% CI: 1.12, 4.62]. In a follow-up study, stratified by smoking status, women in the heart-healthy cluster who had never smoked had a significantly lower risk of carotid stenosis (OR: 0.17; 95% CI: 0.07, 0.36) than did women in the less heart-healthy group at 12-y follow-up (39).

McCullough et al (40) used the 10-item Healthy Eating Index (HEI) (41) to assess the relation between compliance with the USDA’s Dietary Guidelines for Americans (42) and the Food Guide Pyramid (43) and various chronic disease endpoints. Women in the highest quintile of HEI score (which indicated a more desirable eating pattern) had 14% lower CVD risk over 12 y than did women in the lowest HEI quintile. Higher HEI was not associated with lower cancer risk or overall chronic disease risk in these women.

Stampfer et al (44) examined healthy lifestyle behaviors in relation to the development of coronary events (death due to CAD or nonfatal myocardial infarction) in women. Combinations of lifestyle behaviors were based on the following risk factors: diet (low in trans fat and glycemic load; high in cereal fiber, marine n-3 fatty acids, and folate; and a high ratio of polyunsaturated to saturated fat), smoking abstinence, physical activity (≥30 min/d of moderate to vigorous exercise), BMI <25, and alcohol consumption ≥5 g/d. At 14-y follow-up, women with healthier diets (upper 2 quintiles) who did not smoke and who exercised had a reduction of almost 60% in coronary events (RR: 0.43; 95% CI: 0.33, 0.55). The addition of

<table>
<thead>
<tr>
<th>Nutrient intake</th>
<th>Compliant nonsmokers</th>
<th>Compliant smokers</th>
<th>Noncompliant nonsmokers</th>
<th>Noncompliant smokers</th>
<th>p*</th>
<th>p5</th>
<th>p6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>1510.6 ± 38.47</td>
<td>1517.1 ± 33.8</td>
<td>1700.1 ± 27.3</td>
<td>1694.5 ± 22.7</td>
<td>0.96</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Total fat (% of energy)</td>
<td>24.1 ± 0.39</td>
<td>23.7 ± 0.34</td>
<td>35.6 ± 0.28</td>
<td>35.7 ± 0.23</td>
<td>0.83</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Saturated fat (% of energy)</td>
<td>7.3 ± 0.19</td>
<td>7.1 ± 0.17</td>
<td>12.1 ± 0.13</td>
<td>12.0 ± 0.11</td>
<td>0.59</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Polyunsaturated fat (% of energy)</td>
<td>5.6 ± 0.18</td>
<td>5.7 ± 0.16</td>
<td>7.6 ± 0.13</td>
<td>7.6 ± 0.11</td>
<td>0.81</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Monounsaturated fat (% of energy)</td>
<td>8.9 ± 0.18</td>
<td>8.6 ± 0.16</td>
<td>13.2 ± 0.13</td>
<td>13.3 ± 0.11</td>
<td>0.98</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate (% of energy)</td>
<td>57.7 ± 0.58</td>
<td>56.4 ± 0.51</td>
<td>47.4 ± 0.42</td>
<td>45.8 ± 0.35</td>
<td>0.0005</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>18.4 ± 0.31</td>
<td>19.1 ± 0.27</td>
<td>16.9 ± 0.22</td>
<td>17.2 ± 0.18</td>
<td>0.081</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Alcohol (g)</td>
<td>4.2 ± 0.82</td>
<td>7.1 ± 0.72</td>
<td>4.1 ± 0.58</td>
<td>6.7 ± 0.49</td>
<td>&lt;0.0001</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>143.1 ± 8.1</td>
<td>150.1 ± 7.1</td>
<td>228.5 ± 5.7</td>
<td>231.8 ± 4.8</td>
<td>0.46</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Dietary fiber (g)</td>
<td>17.5 ± 0.54</td>
<td>17.9 ± 0.47</td>
<td>15.2 ± 0.38</td>
<td>14.5 ± 0.32</td>
<td>0.35</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>2304.6 ± 77.4</td>
<td>2418.9 ± 68.0</td>
<td>2715.7 ± 55.0</td>
<td>2802.0 ± 45.7</td>
<td>0.105</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Folacin (µg)</td>
<td>289.3 ± 10.6a</td>
<td>302.0 ± 9.3a</td>
<td>263.2 ± 7.5b</td>
<td>240.2 ± 6.2a</td>
<td>0.037</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>136.6 ± 5.3</td>
<td>129.5 ± 4.7</td>
<td>99.4 ± 3.8</td>
<td>97.0 ± 3.1</td>
<td>0.32</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>8.1 ± 0.40</td>
<td>8.0 ± 0.35</td>
<td>9.4 ± 0.28</td>
<td>8.9 ± 0.24</td>
<td>0.28</td>
<td>0.0011</td>
<td></td>
</tr>
<tr>
<td>Vitamin B-6 (mg)</td>
<td>1.8 ± 0.06a</td>
<td>1.9 ± 0.05a</td>
<td>1.7 ± 0.04b</td>
<td>1.6 ± 0.03b</td>
<td>0.068</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>β-Carotene (µg)</td>
<td>4774.2 ± 353.3</td>
<td>4813.4 ± 310.5</td>
<td>4085.7 ± 250.8</td>
<td>3734.3 ± 208.7</td>
<td>0.40</td>
<td>0.0013</td>
<td></td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>672.8 ± 25.6</td>
<td>658.4 ± 22.5</td>
<td>688.7 ± 18.2</td>
<td>658.8 ± 15.1</td>
<td>0.20</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>2743.4 ± 67.9</td>
<td>2747.8 ± 59.6</td>
<td>2552.9 ± 48.2</td>
<td>2533.1 ± 40.1</td>
<td>0.82</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>276.5 ± 7.5</td>
<td>284.5 ± 6.6</td>
<td>265.6 ± 5.3</td>
<td>259.2 ± 4.4</td>
<td>0.76</td>
<td>0.0016</td>
<td></td>
</tr>
</tbody>
</table>

1 The sample includes 1123 women free of cardiovascular disease at baseline with complete data on covariates. Analysis of covariance was used to obtain the age-adjusted means for continuous variables and to identify significant differences. Logistic regression was used to obtain the age-adjusted proportions and to identify subgroups that differ significantly. Means in a row with different superscript letters are significantly different, P < 0.05 (Bonferroni’s corrected t tests).

2 Three-day mean intake met the population-based dietary guidelines from the US Department of Agriculture (USDA) and the American Heart Association (AHA) for coronary artery disease risk reduction for all 3 of the following nutrients: total fat ≤30% of energy (kcal), saturated fat <10% of energy (kcal), and cholesterol <300 mg.

3 Three-day mean intake failed to meet the population-based USDA and AHA dietary guidelines for coronary artery disease risk reduction for ≥1 of the 3 nutrients of interest.

4 The smoking × noncompliance interaction.

5 The main effect of smoking if the smoking × noncompliance interaction was not significant.

6 The main effect of noncompliance if the smoking × noncompliance interaction was not significant.

7 ± SE (all such values).
BMI <25 or both favorable BMI and alcohol intakes to this constellation of risk factors lowered women’s risk of coronary events further (RR: 0.34; 95% CI: 0.23, 0.52 and RR: 0.17; 95% CI: 0.07, 0.41, respectively).

In the current study, FOS Study women who had carotid stenosis at 4-y follow-up had distinct patterns of nutrient intake, including higher total and saturated fat and sodium intakes and lower consumption of fiber, folacin, vitamins C and B-6, potassium, and magnesium. They also had a higher burden of CVD risk factors, including elevated systolic blood pressure, dyslipidemia, diabetes, and smoking exposure. Physical activity levels were similar in women with and without stenosis but were low, on average, in this cohort. Consistent with these data, Kuller et al. (45) showed that the presence of subclinical atherosclerosis in women was associated with modifiable CVD risk factors, including cigarette smoking, higher LDL- and lower HDL-cholesterol concentrations, and higher systolic blood pressure measurement and blood glucose concentrations. From the standpoint of primary CAD prevention, the behavioral and CVD risk factor profiles characteristic of women at risk of subclinical disease suggest certain targets for population-based interventions. They also provide a rationale for more aggressive intervention among those persons at high risk of subclinical disease (45).

The potential complexity of behavioral intervention to promote CAD risk reduction is underscored by these findings. Indeed, about two-thirds of women in the FOS Study population (ie, the diet-noncompliant smokers and nonsmokers) were unable to follow the current population-based dietary guidelines for CVD risk reduction and CAD prevention. The distinct nutrient intake profile of noncompliant women (dietary fat- and sodium-rich and poor micronutrient density) in relation to women with compliant diets points to the potential importance of targeting prevention messages and interventions to the specific lifestyle patterns of women.

Our data suggest that dietary noncompliance and cigarette smoking conferred a similar, 2.5-fold greater risk of stenosis that was independent of other CVD risk factors. Furthermore, the

### TABLE 2
Subject cardiovascular disease (CVD) risk factor characteristics by dietary compliance and smoking status at baseline (1992–1996)

<table>
<thead>
<tr>
<th>CVD risk factor characteristics</th>
<th>Compliant nonsmokers (n = 157)</th>
<th>Compliant smokers (n = 203)</th>
<th>Noncompliant nonsmokers (n = 311)</th>
<th>Noncompliant smokers (n = 452)</th>
<th>P&lt;sub&gt;1&lt;/sub&gt;</th>
<th>P&lt;sub&gt;2&lt;/sub&gt;</th>
<th>P&lt;sub&gt;3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>56.8 ± 9.1&lt;sup&gt;7&lt;/sup&gt;</td>
<td>56.2 ± 9.1</td>
<td>56.1 ± 10.6</td>
<td>54.0 ± 9.2</td>
<td>0.0071</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>124.9 ± 1.4</td>
<td>125.5 ± 1.2</td>
<td>123.5 ± 1.0</td>
<td>123.0 ± 0.8</td>
<td>0.90</td>
<td>0.060</td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>73.0 ± 0.78</td>
<td>72.4 ± 0.68</td>
<td>72.4 ± 0.55</td>
<td>72.2 ± 0.46</td>
<td>0.54</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>26.4 ± 0.42</td>
<td>25.7 ± 0.37</td>
<td>26.6 ± 0.29</td>
<td>26.7 ± 0.25</td>
<td>0.47</td>
<td>0.056</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>207.9 ± 2.7</td>
<td>209.8 ± 2.4</td>
<td>203.3 ± 1.9</td>
<td>208.5 ± 1.6</td>
<td>0.043</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>55.3 ± 1.2</td>
<td>56.0 ± 1.0</td>
<td>57.3 ± 0.85</td>
<td>57.3 ± 0.70</td>
<td>0.81</td>
<td>0.090</td>
<td></td>
</tr>
<tr>
<td>Total:HDL cholesterol</td>
<td>4.0 ± 0.10</td>
<td>4.1 ± 0.09</td>
<td>3.7 ± 0.07</td>
<td>3.9 ± 0.06</td>
<td>0.099</td>
<td>0.0088</td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>125.4 ± 2.5</td>
<td>125.7 ± 2.2</td>
<td>122.5 ± 1.8</td>
<td>125.0 ± 1.5</td>
<td>0.36</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>0.05 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.99</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Cigarette exposure (pack-years)</td>
<td>0.0&lt;sup&gt;6&lt;/sup&gt;</td>
<td>14.6 ± 1.16&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.0&lt;sup&gt;6&lt;/sup&gt;</td>
<td>21.1 ± 0.78&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.0025</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>Physical activity index</td>
<td>33.7 ± 0.40</td>
<td>33.8 ± 0.35</td>
<td>33.7 ± 0.28</td>
<td>33.6 ± 0.23</td>
<td>0.73</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 The sample includes 1123 women free of CVD at baseline with complete data on covariates. Analysis of covariance was used to obtain the age-adjusted means for continuous variables and to identify significant differences. Logistic regression was used to obtain the age-adjusted proportions and to identify subgroups that differ significantly. Means in a row with different superscript letters are significantly different, P < 0.05 (Bonferroni’s corrected tests).

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3 Three-day mean intake failed to meet the population-based USDA and AHA dietary guidelines for CAD risk reduction for ≥1 of the 3 nutrients of interest.

4 The smoking × noncompliance interaction was not significant.

5 The main effect of smoking if the smoking × noncompliance interaction was not significant.

6 The main effect of noncompliance if the smoking × noncompliance interaction was not significant.

7 ± SE (all such values).

### TABLE 3
Odds ratios (ORs) for stenosis in women stratified by dietary compliance and smoking status (1996–1999)

<table>
<thead>
<tr>
<th>Coefficient estimate</th>
<th>P&lt;sup&gt;2&lt;/sup&gt;</th>
<th>OR (95% CI)&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compliant nonsmokers (n = 157)</td>
<td>Reference group</td>
<td>1.00</td>
</tr>
<tr>
<td>Noncompliant nonsmokers (n = 311)</td>
<td>0.9298</td>
<td>0.0171</td>
</tr>
<tr>
<td>Compliant smokers (n = 203)</td>
<td>0.9276</td>
<td>0.0214</td>
</tr>
<tr>
<td>Noncompliant smokers (n = 452)</td>
<td>1.2491</td>
<td>0.0089</td>
</tr>
</tbody>
</table>

1 The sample includes 1123 women free of cardiovascular disease at baseline with complete data on covariates. Dietary compliance is defined as 3-d mean intake that meets the population-based dietary guidelines from the US Department of Agriculture and the American Heart Association for coronary artery disease risk reduction for all 3 of the following nutrients: total fat ≤30% of energy (kcal), saturated fat <10% of energy (kcal), and cholesterol <300 mg. Women who were noncompliant had 3-d mean intakes that failed to meet the guidelines for ≥1 of the 3 nutrients of interest.

2 A formal test for the effect of the smoking status × dietary compliance interaction on stenosis was conducted and yielded P = 0.10.

3 Multivariate logistic regression model adjusted for age, energy intake, systolic blood pressure, total:HDL cholesterol, BMI, and diabetes.
combination of poor eating behavior and smoking appears to be associated with the highest risk of carotid atherosclerosis. Combined lifestyle risks are increasingly recognized (32, 44) but have yet to be formally integrated on a widespread basis into targeted, combined risk-reduction interventions. The high rates of smoking among women with unfavorable dietary patterns also should be recognized and targeted as a potential barrier to changes in nutrition behavior.

The assessment of carotid stenosis by high-resolution B-mode ultrasonography is accurate in diagnosing subclinical CVD (18, 19, 35, 45) and provides a noninvasive method of identifying potential candidates for primary prevention activities. It may also offer an approach for evaluating interventions that are aimed at delaying the development of atherosclerosis (10, 45).

Our observations were established in a cohort of women, most of whom are white residents of a western Boston suburban community. Although this population may somewhat limit the generalizability of our findings, we note that the Framingham Study models for CVD risk have been repeated and confirmed in domestic and international populations (46–49). Our data encourage further research on the relations in women among dietary patterns, subclinical CVD, and other health outcomes.

BEM provided overall direction to this research, including the identification of research questions and hypotheses, review of data analyses, and the development of all aspects of the manuscript, which included the description of the conceptual framework and context of the research, the presentation of results, and the interpretation and discussion of the findings. PAQ was involved in all aspects of this research and manuscript preparation, particularly the presentation of research methods, the results, and their interpretation and the discussion of findings within the context of epidemiologic literature. B-HN and MJP performed all the statistical analyses related to this research and prepared the analytic methods section of this manuscript. JFP was responsible for providing clinical expertise on the use of the carotid stenosis data sets in these analyses, for the presentation of the clinical methods and findings, and for the discussion and interpretation of the stenosis results in this manuscript. RWK was responsible for the review and the development of all aspects of the manuscript in preparation for publication. JMO, as principal investigator of one of the funded research projects that supported this work, provided significant consultation and advice to the research team during the preparation of research for publication. RBBD, as senior biostatistician and head of the Statistical Consulting Unit at the Framingham Study, provided oversight on all aspects of the statistical analyses, the analytic components of this manuscript, and the interpretation of the data. None of the authors had a personal or financial conflict of interest.

REFERENCES
Concordant lipoprotein and weight responses to dietary fat change in identical twins with divergent exercise levels\textsuperscript{1–3}

Paul T Williams, Patricia J Blanche, Robin Rawlings, and Ronald M Krauss

\textbf{ABSTRACT}

\textbf{Background:} Individuals vary greatly in their lipoprotein responses to low-fat diets, with some of this variation being attributable to genes.

\textbf{Objective:} The purpose was to test the extent to which individual lipoprotein responses to diet can be attributed to genes in the presence of divergent exercise levels.

\textbf{Design:} Twenty-eight pairs of male monozygotic twins (one twin mostly sedentary, the other running an average of 50 km/wk more than the sedentary twin) went from a 6-wk 40%-fat diet to a 6-wk 20%-fat diet in a crossover design. The diets reduced fat primarily by reducing saturated and polyunsaturated fat (both from 14\% to 4\%) while increasing carbohydrate intake from 45\% to 65\%.

\textbf{Results:} Despite the twins’ differences in physical activity, the dietary manipulation produced significantly correlated changes ($P < 0.05$) in the twins’ total cholesterol ($r = 0.56$); LDL cholesterol ($r = 0.70$); large, buoyant LDL [Svedberg flotation rate ($S_f$) 7–12; $r = 0.52$]; apolipoprotein A-I ($r = 0.49$); lipoprotein(a) ($r = 0.49$); electrophoresis measurements of LDL-I (LDLs between 26 and 28.5 nm in diameter; $r = 0.48$), LDL-IIB (25.2–24.6 nm; $r = 0.54$), and LDL-IV (22–24.1 nm; $r = 0.50$); and body weight ($r = 0.41$). Replacing fats with carbohydrates significantly decreased the size and ultracentrifuge flotation rate of the major LDL and the LDL mass concentrations of large, buoyant LDL; LDL-I; HDL cholesterol; and apolipoprotein A-I and significantly increased concentrations of LDL-IIA (24.7–25.5 nm) and lipoprotein(a).

\textbf{Conclusions:} Even in the presence of extreme differences in exercise, genes significantly affect changes in LDL, apolipoprotein A-I, lipoprotein(a), and body weight when dietary fats are replaced with carbohydrates.

\textbf{KEY WORDS} Twins, low-fat diet, high-carbohydrate diet, lipoproteins, lipoprotein(a), physical activity, LDL subclasses, apolipoproteins, cholesterol

\textbf{INTRODUCTION}

The risk of coronary heart disease increases in association with higher plasma concentrations of LDL cholesterol, triacylglycerols, and lipoprotein(a) and decreases in association with higher concentrations of HDL cholesterol and apolipoprotein (apo) A-I and with the greater size and buoyancy of the LDL particles (1, 2). Low-fat, high-carbohydrate diets decrease plasma concentrations of LDL cholesterol, HDL cholesterol, and apo A-I and increase lipoprotein(a) and triacylglycerols (3). Low-fat, high-carbohydrate diets also produce a shift in the distribution of LDLs from larger, more buoyant particles to smaller, denser particles (4).

Individuals vary greatly in their lipoprotein responses to low-fat diets, with some of this variation being attributable to genes. Persons having the apo E*4 allele experience greater reductions in LDL cholesterol (5) and large, buoyant LDL [Svedberg flotation rate ($S_f$) 7–12] (6) with low-fat, low-cholesterol diets than do those lacking the allele. Polymorphisms in the apo B gene, the signal peptide insertion allele, the LDL receptor gene, the MN blood group, and the apo A-I promoter region are also reported to affect the LDL response to diet (5). Low-fat diets induce a greater reduction in LDL cholesterol and HDL$_{2b}$, the largest HDL particles, in persons with a genetically influenced profile characterized by a predominance of small LDL particles than in those lacking this trait (7–9).

Studies of monozygotic twins provide evidence of genetic regulation in the absence of prior knowledge of the specific genes involved. Such studies provide a global test for genetic hypotheses while circumventing issues of multiple hypothesis testing that plague exploratory tests of multiple genetic loci (10). For example, overfeeding and caloric expenditure in monozygotic twins causes weight gains and losses that correlate significantly within twin pairs (11, 12). To date, however, only a small proportion of the variation in body weight has been attributed to specific genes (13).

The current study examines the effects of switching from a high-fat, low-carbohydrate diet to a low-fat, high-carbohydrate diet in monozygotic twins to assess the contribution of genes to the diet-induced changes in lipoproteins and body weight. Although it is often difficult to separate the effects of the twins’ shared genotypes from the effects of their shared environment (14), the current design minimizes the effect of the shared environment by 1) deliberately choosing twins with divergent lifestyles (one physically active, one sedentary) and 2) measuring the response to an experimental manipulation of diet (as opposed
\textsuperscript{1}From the Lawrence Berkeley National Laboratory, Donner Laboratory, Berkeley, CA (PTW and RMK), and the Children’s Hospital Oakland Research Institute, Oakland, CA (PJB, RR, and RMK).

\textsuperscript{2}Supported in part by a grant from Dairy Management Incorporated, NIH R01 grant HL072110, and NIH Program Project grant HL-18574 from the National Heart, Lung, and Blood Institute and conducted at Lawrence Berkeley National Laboratory through the US Department of Energy under contract no. DEAC03-76SF00098.

\textsuperscript{3}Address reprint requests to PT Williams, Lawrence Berkeley National Laboratory, Donner Laboratory, 1 Cyclotron Road, Berkeley, CA 94720. E-mail: ptwilliams@lbl.gov.

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to observational twin studies that may be strongly affected by the shared environment).

SUBJECTS AND METHODS

Twenty-nine pairs of male identical twins discordant for exercise participated in a crossover study of high-fat, low-carbohydrate and low-fat, high-carbohydrate diets. The twins were identified among current participants of the National Runners’ Health Study (15) and from announcements distributed at footraces through the Runner’s World race participation program (Rodale Press, Emmaus, PA). Criteria for eligibility were as follows: discordant for exercise (ie, either one twin was sedentary and the other was ran ≥32 km/wk or if both twins ran there was a ≥40-km/wk difference in running distance), no medication use likely to interfere with lipid metabolism, free of chronic disease, nonsmoker, and willingness to abstain from alcohol and follow the prescribed diets over the 12-wk intervention. Each twin completed a questionnaire and signed a consent form approved by the Committee for the Protection of Human Subjects at Lawrence Berkeley National Laboratory, University of California, Berkeley, CA.

The research was conducted in an outpatient setting with careful monitoring of dietary compliance. All participants were counseled by registered dietitians to follow the prescribed diets both before and during the experimental intervention. The twin pairs received, in random order, a 6-wk low-fat, solid-food diet (20% of total energy as fat, 65% as carbohydrates) and a 6-wk high-fat diet (40% fat, 45% carbohydrates) in a crossover design. The 2 experimental diets were designed to achieve a comparison of high and low fat intake by substitution of fat for carbohydrate without significant change in other major nutrients. Nutrient compositions of the diets were calculated by using the Minnesota NUTRITION DATA SYSTEM (NDS) software developed by the Nutrition Coordinating Center (version 4.01; University of Minnesota, Minneapolis, MN). Registered dietitians supplied the participants with personalized menus showing the number and size of servings for the experimental diets.

Seven-day diets were prescribed that represented 95% of the participants’ total caloric intake as estimated from their baseline 4-d food records. The remaining 5% of caloric intake was provided as food combinations that matched the dietary composition of the prescribed diets and that could be consumed ad libitum in response to satiety. The prescribed diets had to be eaten in their entirety within each 7-d period. The 5% additional calories could be consumed as 120 mL (0.5 cup) of low-fat milk with 5 vanilla wafers for the low-fat diet and as 1 teaspoon (∼4 g) of peanut butter with 8 wheat crackers for the high-fat diet. All subjects abstained from alcohol during the study.

The staff contacted the subjects weekly during the study to verify adherence to the diet and to review the protocol. Compliance was assessed by reviewing 4-d diet records and grocery receipts. One twin pair did not complete the dietary intervention.

Twins reported to a local clinic of their choice to have their activity. Plasma was prepared from venous blood collected in tubes containing 1.4 mg Na₂EDTA/mL. Samples were drawn only on Mondays, Tuesdays, or Wednesdays and were shipped overnight on wet ice to ensure that they were delivered to our laboratory by Thursday morning. Before starting the study, all participants received an electronic scale for measuring their own body weight. Height and weight were also measured during the clinic visits.

Lipid and lipoprotein measurements

Fasting plasma lipids were measured at baseline and after each 6-wk diet. Plasma concentrations of total cholesterol and triacylglycerols were measured by enzymatic procedures (ABA 200 instrument, Abbott Laboratories, Abbott Park, IL; 16). HDL cholesterol was measured by the dextran sulfate–magnesium precipitation of apo B–containing lipoproteins followed by enzymatic determination of cholesterol (17, 18). Plasma LDL-cholesterol concentrations were calculated from the formula of Friedewald et al (19). The laboratory remained certified by the Centers for Disease Control and Prevention lipid standardization program throughout the study. Apo A-I and B in plasma was measured by immunoturbidimetric assay (20, 21) with an ITA reagent kit (Bacton Assay Systems Inc, San Marcos, CA). Measurements are performed by using the Express 550 analyzer according to kit instructions. Calibrators and controls are assigned quantitation values based on the International Federation of Clinical Chemistry proposed Standard Reference Material SP1 and by participation in the standardization program directed by the International Federation of Clinical Chemistry and the Centers for Disease Control and Prevention. Intra- and interrun CVs were within ±5%.

Fasting LDL particle diameters and LDL particle subclass intervals based on particle size were calculated from calibration curves by using standards of known size (22). Analyses are based on the area within the LDL-IVB (22.0–23.2 nm), LDL-IVA (23.2–24.2 nm), LDL-IIB (24.2–24.7 nm), LDL-IIIa (24.7–25.5 nm), LDL-IIB (25.5–26.0 nm), LDL-IIA (26.0–26.5 nm), and LDL-I (26.5–28.5 nm) particle size intervals (22, 23). Analytic ultracentrifugation was used to measure concentrations of total lipoprotein mass within multiple regions for dense LDL (S₉, 0–7), buoyant LDL (S₉, 7–12), intermediate-density lipoproteins (IDL; S₉, 12–20) and VLDL (S₉, 20–400) (24).

Statistical analyses

Fifteen pairs started with the high-fat diet and 13 pairs started with the low-fat diet. Because the 2 diet sequences were not equally represented, paired t tests were not used because temporal effects would not be eliminated by the analyses. We therefore computed separately the mean lipoprotein change in switching from a high-fat to a low-fat diet and the mean lipoprotein change in switching from the low-fat to the high-fat diets and their corresponding SEs. We then calculated one-half of the differences of the mean changes and their corresponding SE (one-half of the square root of the sum of the squared SEs) to estimate separately the effect of the diet manipulation on the running twins’ and the sedentary twins’ lipoproteins while eliminating any temporal effects. The difference between the running and the sedentary twins’ dietary responses was calculated by subtracting the lipoprotein change within each twin pair and then analyzing the calculated differences as described above. Because none of the variables responded differently in the running and sedentary twins, we also analyzed the average of the twins’ responses to assess the effect of the diet on lipoproteins with greater statistical power. Twin-pair correlations of the lipoprotein responses to the diets were calculated after adjustment for the diet sequence by
TABLE 1

Baseline screening characteristics of male monozygotic twins according to physical activity

<table>
<thead>
<tr>
<th></th>
<th>Running twins</th>
<th>Sedentary twins</th>
<th>Difference</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Running distance (km)</td>
<td>52.56 ± 20.75</td>
<td>2.39 ± 4.68</td>
<td>50.17 ± 3.77</td>
<td>0.92</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.77 ± 0.06</td>
<td>1.77 ± 0.06</td>
<td>0.00 ± 0.02</td>
<td>0.62</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>73.54 ± 8.64</td>
<td>79.34 ± 11.69</td>
<td>-5.80 ± 1.76</td>
<td>0.47</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.41 ± 1.82</td>
<td>25.35 ± 3.22</td>
<td>-1.94 ± 0.54</td>
<td>0.64</td>
</tr>
<tr>
<td>Apo A-I (g/L)</td>
<td>1.21 ± 0.21</td>
<td>1.11 ± 0.16</td>
<td>0.10 ± 0.03</td>
<td>0.79</td>
</tr>
<tr>
<td>Apo B (g/L)</td>
<td>0.83 ± 0.18</td>
<td>0.92 ± 0.22</td>
<td>-0.09 ± 0.03</td>
<td>0.79</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/L)</td>
<td>0.97 ± 0.51</td>
<td>1.46 ± 0.93</td>
<td>-0.49 ± 0.14</td>
<td>0.57</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.66 ± 0.89</td>
<td>4.74 ± 0.93</td>
<td>-0.08 ± 0.11</td>
<td>0.78</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.32 ± 0.39</td>
<td>1.09 ± 0.3</td>
<td>0.23 ± 0.05</td>
<td>0.76</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.9 ± 0.13</td>
<td>2.98 ± 0.14</td>
<td>-0.08 ± 0.10</td>
<td>0.71</td>
</tr>
<tr>
<td>Lp(a) (µmol/L)</td>
<td>0.6 ± 0.7</td>
<td>0.48 ± 0.53</td>
<td>0.12 ± 0.04</td>
<td>0.99</td>
</tr>
<tr>
<td>LDL peak particle diameter (nm)</td>
<td>26.61 ± 0.86</td>
<td>26.28 ± 0.93</td>
<td>0.33 ± 0.12</td>
<td>0.75</td>
</tr>
</tbody>
</table>

1 Measurements of lipoprotein mass concentrations by use of an analytic ultracentrifuge were not made at the baseline screening visit. n = 28 twin pairs.
Apo, apolipoprotein. Statistical significance was assessed by paired t test or product-moment (Pearson) correlation coefficient.

2 Values are x ± SD.
3 Values are x ± SE.
4 The correlation between the baseline value of the running twin and that of the sedentary twin.
5 P < 0.001.
6 P < 0.005.
7 P < 0.05.
8 P < 0.01.

RESULTS

Baseline

The baseline characteristics of the twins are presented in Table 1. The running twins ran an average of 50 km/wk more than the sedentary twins. Correspondingly, the running twins weighed significantly less than did the sedentary twins, had significantly higher apo A-I and HDL-cholesterol concentrations in plasma, and had significantly lower apo B and triacylglycerol concentrations in plasma. LDL peak particle diameter was also significantly larger in the running twins.

Consistent with their monozygosity, the twins’ heights were significantly correlated (r = 0.92), as were their body masses and body mass indexes. Despite substantial differences in physical activities, the twins exhibited strong, significant correlations for apos A-I and B, triacylglycerols, total cholesterol, HDL cholesterol, LDL cholesterol, lipoprotein(a), and LDL peak particle diameter. Plasma concentrations of LDL-I, LDL-II, LDL-IIIA, LDL-IV, and LDL-IVB were also significantly correlated between the twins.

Switching from the high-fat to the low-fat diet

The nutrient intakes reported on the 7-d food records for the running and sedentary twins during the 2 diets are shown in Table 2. The dietary goals were achieved for both diets. The changes in mean nutrient intake when switching from the high-fat, low-carbohydrate diet to the low-fat, high-carbohydrate diet were not significantly different between the running and sedentary twins for total energy intake (mean change for the running — the mean change for the sedentary twin ± SE: -117.69 ± 92.12 kcal/d), total fat (0.53 ± 0.82%), saturated fat (0.12 ± 0.22%), monounsaturated fat (0.19 ± 0.21%), polyunsaturated fat (0.19 ± 0.49%), carbohydrates (−1.10 ± 1.22%), protein (0.58 ± 0.51%), or dietary cholesterol (5.26 ± 15.21 mg/d).

As shown in Table 3, decreasing dietary fat significantly decreased HDL-cholesterol concentrations in both the running and the sedentary twins. Apo A-I also decreased significantly in the running twins and marginally in the sedentary twins. The decreases in both HDL cholesterol and apo A-I were significant when the running and sedentary twins’ data were averaged, as was the increase in mean plasma lipoprotein(a) concentrations.

Also shown in Table 3 are the changes in VLDL and LDL in response to decreasing fat and increasing carbohydrates. Mean LDL peak particle diameter and the LDL peak floation rate decreased in both the sedentary and the exercising twins. Mass concentrations of large, buoyant LDL also decreased significantly in both groups. Correspondingly, changes in LDL peak particle diameter, LDL peak floation rate, and large, buoyant LDL were strongly significant when the values for the running and sedentary twins were averaged. The additional statistical power when the values for the running and the sedentary twins were averaged resulted in significant decreases being shown in
TABLE 2

Mean nutrient intakes with the high- and low-fat diets of male monozygotic twins according to physical activity.

<table>
<thead>
<tr>
<th></th>
<th>High-fat, low-carbohydrate diet</th>
<th>Low-fat, high-carbohydrate diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Running twins</td>
<td>Sedentary twins</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>2676.8 ± 358.2</td>
<td>2713.5 ± 369.5</td>
</tr>
<tr>
<td>Total fat (% of energy)</td>
<td>39.2 ± 3.0</td>
<td>39.1 ± 3.7</td>
</tr>
<tr>
<td>Saturated fat (% of energy)</td>
<td>12.4 ± 1.2</td>
<td>12.4 ± 0.9</td>
</tr>
<tr>
<td>Monounsaturated fat (% of energy)</td>
<td>12.0 ± 0.7</td>
<td>11.7 ± 0.9</td>
</tr>
<tr>
<td>Polyunsaturated fat (% of energy)</td>
<td>12.2 ± 2.0</td>
<td>12.2 ± 0.6</td>
</tr>
<tr>
<td>Carbohydrates (% of energy)</td>
<td>46.4 ± 3.1</td>
<td>46.6 ± 3.3</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>15.8 ± 0.9</td>
<td>15.6 ± 0.8</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>324.9 ± 58.0</td>
<td>327.1 ± 42.7</td>
</tr>
</tbody>
</table>

All values are ± SD, n = 28 twin pairs. None of the dietary changes were significantly different between the running and the sedentary twins by paired t test.

LDL-I and significant increases in LDL-III A. The decrease in LDL-I and increase in LDL-III A were significant in the sedentary twins but not the running twins (P = 0.10 for LDL-I and P = 0.07 for LDL-III A in the running twins). The lipoprotein responses to the diets were not significantly different between the running and sedentary twins (Table 3).

Concordance within twin pairs

Increased dietary fat did not significantly change body weight (Table 3). However, there was considerable variability in the body weight response to the diets, and the responses were significantly correlated within twin pairs (r = 0.41; Table 3).

TABLE 3

Mean weight and apolipoprotein and lipoprotein concentrations of male monozygotic twins according to physical activity during the 6-wk high-fat and low-fat diets and the changes between diets.

<table>
<thead>
<tr>
<th></th>
<th>Running twins²</th>
<th>Sedentary twins²</th>
<th>Mean change from high-fat to low-fat diet³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High-fat diet</td>
<td>Low-fat diet</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.59 ± 1.50</td>
<td>71.53 ± 1.47</td>
<td>-0.06 ± 0.31</td>
</tr>
<tr>
<td>Apo A-I (g/L)</td>
<td>1.21 ± 0.04</td>
<td>1.13 ± 0.04</td>
<td>-0.08 ± 0.02</td>
</tr>
<tr>
<td>Apo B (g/L)</td>
<td>0.75 ± 0.03</td>
<td>0.76 ± 0.03</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/L)</td>
<td>0.85 ± 0.10</td>
<td>0.84 ± 0.11</td>
<td>0.19 ± 0.06</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.35 ± 0.15</td>
<td>4.19 ± 0.15</td>
<td>-0.16 ± 0.08</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.40 ± 0.08</td>
<td>1.26 ± 0.06</td>
<td>-0.14 ± 0.04</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.57 ± 0.13</td>
<td>2.45 ± 0.12</td>
<td>-0.12 ± 0.07</td>
</tr>
<tr>
<td>ΔLDLc (μmol/L)</td>
<td>0.63 ± 0.14</td>
<td>0.69 ± 0.13</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>ΔLDL peak diameter (nm)</td>
<td>26.85 ± 0.11</td>
<td>26.33 ± 0.14</td>
<td>-0.52 ± 0.10</td>
</tr>
</tbody>
</table>

¹ Values are ± SE. Apo, apolipoprotein; Δ, change; Lp(a), lipoprotein(a); IDL, intermediate-density lipoprotein.

² Values are x ± SE.

³ Values are x ± SE. Significance is from t statistic for the linear contrast between twins who switched from the high-fat to the low-fat diet and those who switched from the low-fat to the high-fat diet.

⁴ The correlation between the responses of the running twins and those of the sedentary twins, adjusted for the diet sequence.

⁵ P < 0.05.

⁶ P < 0.001.

⁷ P < 0.01.

⁸ P < 0.005.
Despite the substantial differences in physical activity, changes in apo A-I were strongly correlated within twin pairs, as were changes in lipoprotein(a) (Table 3).

The strongest correlation between the running and sedentary twins’ lipoproteins was the correlation in the LDL-cholesterol response when switching from a high-fat to a low-fat diet (Figure 1). The data shown in Table 3 suggest that the within-pair correlation for changes in LDL cholesterol reflects within-pair concordant changes in LDL-I and the most buoyant LDL (Sf 7–12). Changes in LDL-IIB and LDL-IV were also significantly correlated between the running twins and the sedentary twins (Table 3).

The correlation between the twins’ lipoprotein changes could not be attributed to concordance in their adherence to the dietary protocol. The correlations for changes in percentages of energy from protein and carbohydrate and dietary cholesterol were all nonsignificant (0.06 ≤ r ≤ 0.08) when switching from the high-fat, low-carbohydrate diet to the low-fat, high-carbohydrate diet. One of the twin pairs reported concordantly low changes in total and saturated fat intakes and one of the other twin pairs reported concordantly low changes in polyunsaturated fat intake. When we excluded these 2 twin pairs, the significant twin correlation was eliminated between changes in total percentage fat intake (r = 0.36 reduced to r = −0.15), percentage saturated fat intake (r = 0.58 reduced to r = 0.14), percentage monounsaturated fat intake (r = 0.36 reduced to r = 0.18), and percentage polyunsaturated fat intake (r = 0.36 reduced to r = −0.13) when switching between diets. Eliminating these 2 twin pairs had almost no detectable effect on the twin correlations for changes in apo A-I (r = 0.47), total cholesterol (r = 0.56), LDL cholesterol (r = 0.70), lipoprotein(a) (r = 0.47), LDL-I (r = 0.40), LDL-IIB (r = 0.57), LDL-IV (r = 0.50), LDL-IVB (r = 0.49), or large, buoyant LDL mass (r = 0.58) after switching from the high-fat, low-carbohydrate diet to the low-fat, high-carbohydrate diet.

**DISCUSSION**

The lipoprotein changes shown in these 28 twin pairs confirm previous reports by ourselves and others that switching from a high-fat, low-carbohydrate diet to a low-fat, high-carbohydrate diet decreases HDL cholesterol and apo A-I and increases lipoprotein(a) (25–27). The diet also decreased the size and buoyancy of the LDL particle distribution as a result of reductions in LDL particles of Sf 7–12 and 26–28.5 nm diameter (LDL-I). In addition, gradient gel electrophoresis showed significant increases in LDL-III. As shown in Table 3 and Figure 1, much of the LDL response in switching from a high-fat, low-carbohydrate diet may be accounted for by genes.

Whereas our previous studies held total caloric intake constant or manipulated calorie intake to hold body weight constant (4, 6, 7, 8), here we prescribed 95% of caloric intake and allowed each subject to supplement his diet with food combinations in accordance with individual preferences to achieve satiety while maintaining the nutrient composition of the diets. This method more realistically reflects the implementation of these diets in free-living unsupervised populations. This approach was taken because weight and lipoprotein changes that occur with real-life exposure to these diets may differ from those observed when caloric intake or body weights are forced to remain constant. For example, reductions in dietary fat have been reported by others to increase triacylglycerol and the ratio of total cholesterol to HDL cholesterol under weight-maintenance conditions but not under ad libitum conditions leading to weight loss (28).

The unique study design showed significant within-pair correlations in the twins’ lipoprotein responses to the dietary manipulations despite their divergent lifestyles. The strongest correlation was for changes in LDL cholesterol (Figure 1). Although several genes have been linked to LDL-cholesterol change during dietary manipulation (5), these are unlikely to account for the 49% of the variance in LDL-cholesterol change that our study attributed to the twins’ genes or shared environment. Analytic ultracentrifugation and gradient gel electrophoresis suggest that the concordance in the twins LDL-cholesterol response involves large, buoyant LDL particles of Sf 7–12 and large LDL particles of the LDL-I subclass. The agreement among 3 independent LDL measurements involving 3 separate methods confirms the concordant LDL-cholesterol response to the diet.

Diet-induced changes in the LDL-IIB subclass were also significantly correlated within twin pairs, as were changes in LDL-IV. The LDL-I/B subclass is a relatively minor portion of the LDL distribution that was recently shown to have an independent association with coronary disease progression (29). The discontinuity in the concordance of the monozygotic-twin diet response between LDL-IIB and LDL-IV shown in Table 3 is similar to the discontinuities we reported when LDL subclasses are correlated with atherosclerosis (29) and other lipoproteins (30).

The high monozygotic correlation for lipoprotein(a) measured cross-sectionally is consistent with the finding that >90% of the variation in lipoprotein(a) concentrations is accounted for by the apolipoprotein(a) gene (31). Our data (Table 3) also suggest a strong genetic influence on the lipoprotein(a) response to diet.

We recognize that free-living populations could be less likely to follow controlled diets than are subjects for whom food is supplied. However, we have now completed several studies of men and women with similar dietary protocols (4, 6, 7, 9). Our success in implementing these studies is reflected both in diet records and by the finding that mean lipid responses conform with those predicted from previous controlled feeding studies (32).

We defined divergent lifestyles with respect to different levels of physical activity. As shown in Table 1, runners weighed significantly less than did their sedentary twins, had lower plasma

**FIGURE 1.** Changes (Δ) in plasma LDL-cholesterol concentrations when switching from a 6-wk high-fat diet (40%) to a 6-wk low-fat diet (20% fat) in 28 monozygotic twin pairs discordant for physical activity. The diagonal is not a line fitted to the observations but rather a line drawn as a reference to the locus of points where the changes are identical in the twin pairs. r = 0.70, P < 0.0001.
concentrations of apo B and triacylglycerols, higher plasma concentrations of apo A-I and HDL cholesterol, and larger LDL peak particle diameter. Although these lipoprotein and weight differences are well documented between vigorously active and inactive men (33–35), the data in Table 1 show that these differences persist when genetic effects are controlled for, which is an important consideration because the lipoprotein response to exercise is affected by genes (36). Genes presumably also partially explain why sedentary men with high HDL-cholesterol concentrations run longer weekly distances when enrolled in a training program than do those with low HDL-cholesterol concentrations (37, 38). The running twins also had higher concentrations of lipoprotein(a) than did their sedentary brothers, which has not been consistently observed by others (39–41) but may have been discernible in our study design because we matched for genotype [ie, Table 1 shows a strong genetic concordance for lipoprotein(a) values].

Our results suggest there are genes that strongly influence the LDL-cholesterol response to diet, even in the presence of large differences in physical activity. These genes appear to primarily affect the dietary response of the larger, more buoyant LDL particles. Previous studies have indicated that these particles are more strongly associated with changes in saturated fat intake than are other LDL species (42). Even the most physically active men are susceptible to the effects of diet on HDL-cholesterol, apo A-I, and large, buoyant LDL concentrations and the size and buoyancy of the predominant LDL particles. The prominent role genes play in regulating lipoprotein response to diet is evident whether following ab libitum dietary choices (Table 1) or large dietary perturbations in carbohydrate and fat consumption, regardless of the level of physical activity (Table 3). Moreover, our analyses support earlier observations indicative of the genetic regulation of weight change after environmental perturbation (11, 12). On the basis of these results, we believe that detailed analyses using genetic association or linkage studies are warranted to identify the causes of the associations of diet with lipoprotein and weight.

We acknowledge the valuable contribution of Susan Fernstrom for planning and implementing the dietary intervention and for dietary assessment. PTW designed the study, directed the recruitment, analyzed the data, and wrote the manuscript. PJB directed the lipoprotein analyses, RR directed the intervention and coordinated the collection of laboratory samples, and RMK secured funding, was the principal investigator of the study, helped design the study, and contributed critically to the scientific content of the manuscript.

REFERENCES


Maternal methylenetetrahydrofolate reductase deficiency and low dietary folate lead to adverse reproductive outcomes and congenital heart defects in mice¹⁻³

Deqiang Li, Laura Pickell, Ying Liu, Qing Wu, Jeffrey S Cohn, and Rima Rozen

ABSTRACT

Background: Genetic or nutritional disturbances in folate metabolism may affect embryonic development because of the critical role of folate in nucleotide synthesis and methylation reactions. The possible role of a mild deficiency in methylenetetrahydrofolate reductase (MTHFR) and low dietary folate in pregnancy outcomes and heart morphogenesis requires further investigation.

Objective: We investigated the effect of mild MTHFR deficiency, low dietary folate, or both on resorption rates, on length and weight, and on the incidence of heart malformations in murine embryos.

Design: Female Mthfr¹/² and ¹/⁻ mice were fed a control diet (CD) or a folic acid–deficient diet (FADD) before mating with male Mthfr¹/⁻ mice. On gestational day 14.5, implantation and resorption sites were recorded and viable embryos were examined for gross malformations, growth delay, and congenital heart defects.

Results: Plasma homocysteine in Mthfr¹/⁻ dams and in FADD-treated dams was significantly higher than that in Mthfr¹/² dams and CD-treated dams, respectively. A significantly higher rate of resorption and greater developmental delay were observed in hyperhomocysteinemic mice than in CD-treated ¹/² dams. Heart defects were identified in 4 of 11, 5 of 10, and 4 of 10 litters from CD-treated ¹/², FADD-treated ¹/², and FADD-treated ¹/⁻ dams, respectively, but not in any of those from CD-treated ¹/² dams (0/11 litters).

Conclusion: Our findings suggest that mild MTHFR deficiency, low dietary folate, or both in the dams increase the incidence of fetal loss, intrauterine growth retardation, and heart defects. These data support the benefit of folic acid supplementation in pregnant women, particularly in those with MTHFR deficiency.

KEY WORDS—Folate, homocysteine, methionine, methylenetetrahydrofolate reductase, MTHFR, pregnancy complications, intrauterine growth retardation, fetal loss, congenital heart defects

INTRODUCTION

Folate derivatives are involved in a variety of important cellular reactions, including nucleotide synthesis and the generation of methionine and S-adenosylmethionine (SAM) in the methylation cycle. Methylene-tetrahydrofolate reductase (MTHFR), a key enzyme in these pathways, catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a major carbon donor for methionine synthesis from homocysteine. Cloning of MTHFR (1) led to the identification of a common variant (677C→T; A222V) that results in a thermolabile enzyme with only 35–50% residual activity in homozygous mutant (TT) persons. This prevalent genotype (10–15% of many North American and European populations) is the most common genetic risk factor for hyperhomocysteinemia (2). Hyperhomocysteinemia, or low methionine synthesis, which results from mild MTHFR deficiency or inadequate dietary folate, may contribute to several pathologic states through various mechanisms such as direct toxic effects or through indirect effects such as a disruption in methylation or an increase in oxidative or endoplasmic reticulum stress (3–7). The well-studied pathologic states associated with homocysteine metabolism include vascular diseases and neural tube defects (4–6, 8).

A few clinical reports suggested that the common MTHFR polymorphism or low dietary folate may increase the risk of intrauterine growth retardation (IUGR), fetal loss, and other pregnancy complications (9–12); other studies did not show these associations (13–16). The relation between folate and congenital heart defects (CHDs), one of the most common types of birth defects, is also unclear. Two studies that examined the effect of maternal MTHFR mutant genotype on offspring concluded that it was associated with increased risk of heart defects (17, 18), whereas one study of both maternal and offspring genotypes did not reach this conclusion (19).

To investigate the potential influence of MTHFR deficiency and dietary folate on reproductive outcomes and CHDs, we chose a different approach—the investigation of an animal model of mild MTHFR deficiency that had been generated in our laboratory (20). Only a few animal studies have been conducted on the effects of dietary folate deficiency on reproductive outcomes (21, 22), and none have thoroughly investigated CHD or the role of Mthfr. Mthfr¹/⁻ mice have a high mortality in the first few weeks of life, whereas mice with a single knockout allele (ie,
Mhfr+/− are phenotypically normal. The latter mice represent a good animal model for mild MTHFR deficiency in humans because they have ~60% residual MTHFR activity and plasma homocysteine concentrations that are ~1.6 times higher than those of their wild-type (Mhfr+/+) littermates (20). Mhfr+/− mice also have low methylation potential (20) and were recently shown to have impaired acetylcholine-induced arterial relaxation (23). These findings have all been reported in clinical studies of hyperhomocysteinemia or mild MTHFR deficiency (24, 25), which supports the validity of this animal model for studies of the clinical disorders associated with disturbed folate or homocysteine metabolism. In the current study, we used these Mhfr-deficient mice to examine the effect of MTHFR deficiency, in the presence and absence of low dietary folate, on pregnancy complications and heart defects.

MATERIALS AND METHODS

Mice and diets

Animal experimentation was approved by the Montreal Children’s Hospital Animal Care Committee, according to the guidelines of the Canadian Council on Animal Care. Mhfr knockout mice were generated as reported (20) and backcrossed for homocysteine concentrations that are because they have a good animal model for mild MTHFR deficiency in humans. Mice were generated as reported (20) and backcrossed for homocysteine concentrations that are because they have a good animal model for mild MTHFR deficiency in humans. Mice were housed overnight in plastic-bottomed cages embedded with shavings and were exposed to a 12-h light-dark cycle in a temperature-controlled room. After weaning, female mice were placed on two-amino acid-defined diets (Harlan Teklad, Indianapolis, IN) with all the necessary components recommended by the American Institute of Nutrition (26). A control diet (CD) contained the recommended amount of folic acid for rodents (2 mg/kg diet), and the folic acid–deficient diet (FADD) contained 0.3 mg folic acid/kg diet, which is ~14.3% of the amount of folic acid in the CD. Both diets contained 1% succinyl sulfaflavozole, an antibiotic that is used to prevent the generation of folate by intestinal bacteria (27). After 6 wk of their allotted diets, female mice were housed overnight with Mhfr+/− males (aged 80–100 d). Males were fed standard rodent chow (Agribbrands Purina, St-Hubert, Canada) while they were in their own cages. The next morning, females were checked for the presence of vaginal plugs. If plugs were present, that morning was designated as gestational day (GD) 0.5. Females were fed their designated diets during the breeding period and throughout pregnancy until killed. On GD 14.5, pregnant mice were asphyxiated with carbon dioxide, and blood was collected by cardiac puncture. Resorption sites were identified by the smaller size and more necrotic hemorrhagic appearance than were seen in normal embryos and placentas. Resorption rate was calculated as the ratio of resorption sites to the total number of implantation sites (28). All embryos present were dissected and examined for gross malformations, and crown-rump lengths and individual weights were measured. Developmental delay was assessed by examining the morphology of individual viable embryos and staging them according to their resemblance to normal embryos on a given GD (29). The morphologic examination of embryos was completed by a single person, who was blinded to the litters. Embryos were then fixed in 4% paraformaldehyde overnight and transferred to 70% ethanol for storage. Their yolk sacs were dissected and washed thoroughly in phosphate-buffered saline for subsequent embryonic Mhfr genotyping.

Plasma amino acid analysis

Blood was collected into tubes containing EDTA. Plasma was immediately separated from blood by centrifugation at 4000 × g for 7 min at 4 °C. Plasma was then frozen on dry ice and stored at −70 °C. Plasma total homocysteine (tHcy) and methionine were assayed with the use of HPLC as described previously (30, 31).

Mhfr genotyping

Genomic DNA from maternal toes or livers and from embryonic yolk sacs was isolated by using phenol-chloroform extraction. Polymerase chain reaction was performed according to previously described protocols (20).

Histologic tests

Embryos were processed through a series of ethanol, xylene, and paraffin incubations, which took 45 min per step. They were then embedded and sectioned serially in 10-μm transverse sections. All sections were examined under bright-field illumination with the use of an inverted microscope. Selected sections were stained with hematoxylin and eosin and then photographed.

Statistical analysis

The dam or the litter was considered as the unit for statistical analysis. For parametric data, results were expressed as mean ± SEM and examined by two-factor analysis of variance (ANOVA). For nonparametric data, the Kruskal–Wallis test and two-factor ANOVA of the ranks were used to determine significance for multiple comparisons of resorption rate, percentage of delayed embryos, and incidence of CHD in each litter. Analyses of the contribution of the embryonic genotype to embryonic delay and CHD were conducted by using the two-tailed Fisher’s exact test. All statistical analyses were performed with SPSS for WINDOWS software (version 11.0; SPSS Inc, Chicago, IL). P values < 0.05 were considered significant.

RESULTS

Plasma homocysteine and methionine concentrations

On GD 14.5, mean plasma tHcy in CD-treated +/+ and FADD-treated dams (both +/+ and +/−) was significantly higher than that in CD-treated +/+ dams (control group), which was 13.81 ± 1.55 μmol/L. The highest concentrations of homocysteine (50.66 ± 1.81 μmol/L) were seen in the FADD-treated +/− dams (Figure 1). The overall effects of genotype and diet were highly significant, and there was a marked interaction (P < 0.01, two-factor ANOVA). Plasma tHcy was significantly higher in Mhfr +/− dams than in Mhfr +/+ dams, and there were significant genotype effects in both dietary groups. FADD-treated dams had significantly higher tHcy than did CD-treated dams. These results indicate that the folate-deficient diet had produced the desired increase in tHcy, and they confirm our previous finding that the Mhfr +/− genotype is associated with mild or moderate hyperhomocysteinemia (20).

Plasma methionine concentrations in CD-treated +/+ dams and FADD-treated dams (both +/+ and +/−) were lower than those in CD-treated +/+ dams (control group), although the differences due to genotype or diet were not significant (two-factor ANOVA) (Figure 2). There was no significant genotype × diet interaction.
Reproductive outcomes

No significant differences were found in mean weights of female mice before mating (data not shown). CD-treated +/− dams and FADD-treated dams (+/+ and +/−) had significantly higher resorption rates on the Kruskal-Wallis test than did the control group. The resorption rate in the control group (CD +/+ dams) was 13.37 ± 3.02%, a value that is similar to that previously reported in BALB/c mice (32). Mthfr genotype had a significant effect on embryonic resorption, with a resorption rate in +/− dams that was twice that in +/+ dams on the control diet. The low-folate diet had a particularly marked effect on resorption: ~50% of embryos from FADD-treated dams (both +/+ and +/−) died in utero by GD 14.5. This dietary effect was significant in both genotype groups. Two-factor ANOVA of the ranks found significant overall effects of genotype (P < 0.05) and of diet (P < 0.05) on resorption. However, the effect of genotype in the FADD group was minimal; Mthfr +/− dams showed a rate of resorption only 15% higher than that in Mthfr +/+ dams. The effect of the Mthfr genotype may be relatively minor under circumstances of strong dietary stress.

Examination of the gross morphology of viable embryos did not find obvious anomalies in any group. However, an overall developmental delay (from a half-day up to 2 d) was observed in some embryos, which suggests IUGR. When comparing the percentage of delayed embryos between groups (Kruskal-Wallis test), we found that significantly more embryos with development delays were derived from CD-treated +/− and FADD-treated dams (+/+ and +/−) than from the control group (Table 1). Mthfr genotype had a significant effect on delay in dams on the CD, but not in dams on the FADD. This pattern is similar to that seen for the rate of resorption, which again suggests that the dietary influence may minimize or completely mitigate the effect of genotype. The FADD was associated with a proportion of delayed embryos of almost 20%, but the dietary effect was significant only in the +/− group.

We also examined the overall genotype and dietary effects for the percentage of delayed embryos by using two-factor ANOVA of the ranks (Table 1). The overall dietary effect was significant, but genotype effect was not; there were no significant interactions between genotype and diet. This observation is consistent with our hypothesis that the genotype may have little or no effect in conditions of severe dietary stress.

Average embryonic crown-rump length and weight were smaller in dams of the Mthfr +/− genotype and in dams fed FADD (Table 1), findings that are consistent with the results for delay. The dietary effect was significant, but the effects of genotype on length and weight had only borderline significance (P = 0.09 and 0.06, respectively). There was no significant diet × genotype interaction for length or weight (two-factor ANOVA).

Cardiac malformations in embryos

Viable embryos were sectioned for anatomical and histologic analyses. As shown in Table 2, cardiac malformations were not detected in any of the 78 embryos from 11 litters of CD-treated +/+ dams. On the other hand, 4 of 11 litters (4/62 embryos) from CD-treated +/− dams, 5 of 10 litters (9/39 embryos) from FADD-treated +/+ dams, and 4 of 10 litters (6/37 embryos) from FADD-treated +/− dams showed heart defects. The Mthfr mutant genotype was associated with a significant increase in heart defects in dams on the CD, whereas genotype did not influence rate of CHD in mice on the FADD. There was a significant dietary effect on embryos from Mthfr +/+ dams but not on embryos from Mthfr +/− dams. These results were obtained by using the Kruskal-Wallis test.

To examine overall effects of genotype and diet, we also performed a two-factor ANOVA (Table 2). The dietary effect was significant for incidence of CHD, whereas there was no overall difference for genotype or for the diet × genotype interaction. As mentioned earlier, the genotype effect may be swamped by the severe dietary stress.

Most of the embryos with heart defects (14 of 19) had isolated ventricular septal defects (VSDs) (Figure 3e), whereas 2 had double-outlet right ventricle (DORV) (Figures 3f and 3g), and 3 had endocardial cushion defects (ECDs) in conjunction with DORV (Figure 3h). These embryos also had variably thinner...
myocardia than did embryos from CD-treated +/+ dams (Figure 3h). The 3 embryos with ECD and DORV also had congestive heart failure, as shown by the dilation of vena cava and atria, and liver congestion (see Figure 4c and d, respectively).

### Influence of embryonic genotypes

The Mthfr genotype distribution in viable embryos from each group is shown in Table 3. The ratio of embryonic Mthfr genotypes (+/+, +/−, and −/−) in each group of dams was not significantly different from the expected Mendelian ratios. These data suggest that there is no significant loss of homozygous mutant mice during gestation. We did not observe significant changes in the incidence of embryonic delay or CHDs between embryos of different genotypes within each group of dams. However, this could be due to the small numbers of embryos in each genotype group.

### DISCUSSION

Our results suggest that Mthfr deficiency in mice increases the incidence of pregnancy loss, fetal growth retardation, and CHDs. The conclusions from clinical studies have not been consistent with respect to the role of MTHFR in these types of complications (9–19). Some of the limitations in the clinical studies were the small sample sizes, the lack of information on dietary folate, and the mixed ethnic background of the subjects. The availability of an animal model with MTHFR deficiency has allowed us to overcome most of these potential confounders.

A common link between MTHFR deficiency and folic acid deficiency is an elevation in plasma homocysteine. The influence of MTHFR deficiency on plasma homocysteine was shown in numerous clinical studies and in our original report on Mthfr-deficient mice (2, 20). This study confirms that Mthfr-deficient mice have elevated plasma homocysteine and that dietary folate deficiency raises plasma homocysteine to an even greater extent. The genetic and dietary folate deficiencies appear to have additive effects on plasma homocysteine, and each component contributes to an increase of ≈15 μmol homocysteine/L. The amount of folic acid in our well-defined CD was the required amount for rodents. However, the plasma homocysteine in mice fed our CD was significantly higher than that previously reported for these mice fed laboratory chow (20), because chow contains folate concentrations that are 3-fold the recommended amount.

### TABLE 2

Effects of Mthfr deficiency and folate deficiency on congenital heart defects in viable embryos.

<table>
<thead>
<tr>
<th>Types of CHD</th>
<th>Control diet</th>
<th>Folic acid–deficient diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable embryos (n)</td>
<td>78</td>
<td>62</td>
</tr>
<tr>
<td>Viable embryos with CHD (n)</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Affected litters [n (total n)]</td>
<td>0 (11)</td>
<td>4 (11)</td>
</tr>
<tr>
<td>Incidence of CHD per litter (%)</td>
<td>0</td>
<td>7.12 ± 3.05 3,7</td>
</tr>
<tr>
<td>Types of CHD</td>
<td>22.5 ± 9.94 7</td>
<td>± 12.17 ± 6.17 7</td>
</tr>
<tr>
<td>Isolated VSD</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>DORV</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>DORV+ECD</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

1 CHD, congenital heart defects; VSD, ventricular septal defect; DORV, double-outlet right ventricle; ECD, endocardial cushion defect. Two-factor ANOVA of the ranks indicated a significant overall dietary effect for incidence of CHD (P < 0.05). There was no significant overall genotype effect and no significant diet × genotype interaction.

2 SEM (all such values).

3 Significantly different from Mthfr +/+ dams fed the control diet, P < 0.05 (Kruskal-Wallis test).
Our findings support an association between hyperhomocysteinemia and adverse pregnancy outcomes, as reported previously (11, 33). Whether homocysteine is an independent teratogen or a biomarker for disturbed folate metabolism or methylation remains a controversial topic. Some in vitro evidence supports the former argument—that high homocysteine alone is embryotoxic—in a dose-dependent manner (34, 35). The precise pathogenic mechanism is unknown, but hyperhomocysteinemia might lead to placental vasculopathy, because hyperhomocysteinemia is known to be toxic to the vasculature. This vasculopathy could result in placental infarcts, which would compromise the uteroplacental circulation and reduce fetal blood supply and thus lead to IUGR and spontaneous abortion (33). Examination of the placenta in hyperhomocysteinemic states might prove useful in addressing this question. An alternative mechanism could be that homocysteine results in an increase in S-adenosylhomocysteine (SAH), which can inhibit methylation reactions that are dependent on SAM. Altered DNA methylation was shown in hyperhomocysteinemia and in MTHFR deficiency (20, 24, 36). Disturbances in DNA methylation are associated with changes in gene expression, which could affect the growth and development of embryos. Folate deficiency with hyperhomocysteinemia may also result in imbalances in nucleotide pools and may affect DNA synthesis or DNA repair (3, 33).

The 3 groups of mice with hyperhomocysteinemia had significantly higher rates of adverse pregnancy outcomes than did the control group (CD-treated+/+ dams). However, we observed a resorption rate in FADD-treated+/+ dams (48.9%) that was almost twice that in CD-treated+/+ dams (27.9%), despite relatively similar homocysteine values (25.5 μmol/L and 28.9 μmol/L, respectively). Furthermore, FADD-treated Mthfr+/+...
and +/− dams had similar resorption rates, whereas the +/+ − dams had homocysteine concentrations twice those of the +/+ − dams (50.6 and 25.5 μmol/L, respectively). These observations suggest that maternal plasma homocysteine may not be the only etiologic factor. Other compounds in folate metabolism—eg, methionine or SAM concentrations, nucleotide pools, or methylation intermediates—might be involved and could provide a better correlation with outcomes. Plasma methionine concentrations were lower in +/+ − dams and in FADD-treated dams than in CD-treated +/+ − dams, although the differences were not significant. Plasma homocysteine and methionine concentrations, although useful, may not accurately reflect the concentrations of these compounds in tissues. Changes in intracellular metabolites or alterations in methylation reactions could account for the delay in embryonic development as well as for the fetal loss in maternal +/+ − or folate deficiency, particularly because extreme IUGR may lead to fetal death (33).

Folate deficiency and MTHFR deficiency are well-characterized risk factors for neural tube defects, and studies of other types of birth defects are now emerging (37). The clinical literature on CHDs is consistent with a preventive role of folate supplementation (38–40), but studies of MTHFR in heart defects are sparse and inconclusive (17–19). A low-folate diet administered to mice resulted in a delay of conotruncal septation, a process. Multiple cell types are involved, each of which needs to be restricted to a single cell type, because we also observed cardiomyopathy and a range of CHDs, including ECDs, that involve mesenchymal cells.

Although it is not clear whether hyperhomocysteinemia or its consequences affect heart development, we observed that the incidence of CHDs in FADD-treated +/+ − dams was higher than that in CD-treated +/+ − dams, despite relatively similar homocysteine concentrations. This observation provides another suggestion that homocysteine may not be the only teratogen or that plasma homocysteine does not accurately reflect intracellular metabolism or toxicity.

Some embryos with severe CHDs also had heart failure, as manifested by venous congestion and liver congestion. These embryos were likely to die in utero. It is also possible that some resorbed embryos had CHD alone or with other birth defects, and that they died as a result. Because of the potential for contamination with maternal tissue, we could not examine the genotypes of resorbed embryos. However, among viable embryos, the distribution of +/+ − genotypes was close to the expected Mendelian ratio in each group, which suggested that there was no increased loss of +/+ − deficient embryos. Moreover, there appeared to be no consistent correlation between embryonic +/+ − genotype and the incidence of developmental delay or CHDs in each group, although our numbers were too small for definitive conclusions. Our findings therefore suggest that the embryonic +/+ − genotype may have less of a role than does maternal genotype in the determination of pregnancy outcomes and some birth defects. On GD 14.5, most embryonic organs are well developed, but they still rely on the maternal circulation for nutritional components, including folate. Thus, it is not surprising that the disruption of the maternal folate metabolism by genetic or nutritional means would affect embryonic folate supply. In addition, maternal hyperhomocysteinemia could, because of genetic or nutritional folate deficiency, result in embryonic hyperhomocysteinemia through the uteroplacental circulation (33).

Clinical studies have suggested that MTHFR genotype and folate status may have an interactive effect, at least with respect to plasma homocysteine (43). In the current study, we observed this interaction with respect to homocysteine but not with respect to outcomes, because we did not observe significant differences in resorptions, delays, or heart defects between FADD-treated +/+ − and +/+ − dams. The FADD we used is already very severe and may mitigate the effects contributed by +/+ − deficiency. A diet with a smaller decrease in folate concentrations might have allowed us to observe an interactive effect.

### TABLE 3

<table>
<thead>
<tr>
<th>Dam genotype</th>
<th>Control diet</th>
<th>Folic acid–deficient diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Embryo</td>
<td>Embryo</td>
<td>Embryo</td>
</tr>
<tr>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Embryo</td>
<td>Embryo</td>
<td>Embryo</td>
</tr>
<tr>
<td>+/+</td>
<td>+/+</td>
<td>−/+</td>
</tr>
<tr>
<td>Embryo</td>
<td>Embryo</td>
<td>Embryo</td>
</tr>
<tr>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Embryo</td>
<td>Embryo</td>
<td>Embryo</td>
</tr>
<tr>
<td>+/+</td>
<td>+/+</td>
<td>−/+</td>
</tr>
<tr>
<td>Embryo</td>
<td>Embryo</td>
<td>Embryo</td>
</tr>
</tbody>
</table>

Viable embryos (n) | 43 | 35 | 20 | 31 | 11 | 16 | 23 | 9 | 20 | 8

Delayed embryos [n (%)] | 2 (4.65) | 0 (0) | 2 (10.00) | 3 (9.68) | 2 (18.18) | 3 (18.75) | 4 (17.39) | 1 (11.11) | 5 (25.00) | 1 (12.50)

Embryos with CHD [n (%)] | 0 (0) | 0 (0) | 2 (6.45) | 2 (18.18) | 2 (12.50) | 7 (30.43) | 1 (11.11) | 4 (20.00) | 1 (12.50)

† CHD, congenital heart defects. There was no significant effect of embryonic genotype on embryonic delay or congenital heart defects within each group of dams (Fisher’s exact test).
In conclusion, the current study found that genetic and dietary disruptions in folate metabolism led to adverse pregnancy outcomes and CHDs. These findings, which were consistent with the multifactorial inheritance model for these disorders, suggested that folate supplementation for pregnant women may be important in the prevention of these common conditions, particularly in women with mild MTHFR deficiency.

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DL, LP, and RR contributed to the design of the experiment, analysis of data, and writing of the manuscript. DL, LP, YL, QW, and JSC contributed to the collection of data. The authors had no financial or personal conflicts of interest.

REFERENCES

The Ala54Thr polymorphism of the fatty acid–binding protein 2 gene is associated with a change in insulin sensitivity after a change in the type of dietary fat1–3

Carmen Marín, Francisco Pérez-Jiménez, Purificación Gómez, Javier Delgado, Juan Antonio Paniagua, Aquiles Lozano, Begoña Cortés, Yolanda Jiménez-Gómez, María José Gómez, and José López-Miranda

ABSTRACT
Background: Insulin resistance, a condition associated with type 2 diabetes, results from the interaction of environmental and genetic factors.
Objective: We examined the influence of the intestinal fatty acid–binding protein 2 (FABP2) Ala54Thr polymorphism on insulin sensitivity.
Design: Fifty-nine healthy young subjects (28 were Ala54/Ala54, 27 were Ala54/Thr54, and 4 were Thr54/Thr54) completed 3 diets, each of which lasted 4 wk. The first diet, which all subjects consumed, was a high–saturated fatty acid (SFA) diet (38% of energy as fat and 20% of energy as SFAs). The second and third diets were administered according to a randomized crossover design, and they consisted of a low-fat and high-carbohydrate diet (CHO diet; 28% of energy from fat and <10% of energy from SFAs) and a high–monounsaturated fatty acid (MUFA) diet (called the Mediterranean diet; 38% of energy from fat and 22% of energy from MUFAs). All food and drinks were prepared and provided in the research kitchen. We determined insulin sensitivity in vivo by using the insulin suppression test with somatostatin.
Results: Steady state plasma glucose concentrations were significantly higher in Ala54Thr subjects after the SFA diet than after the CHO diet or the Mediterranean diet. The plasma free fatty acid concentrations in these subjects were significantly lower after the CHO diet and the Mediterranean diet than after the SFA diet. However, no significant differences between the 3 diets were observed in the Ala54 allele homozygotes.
Conclusion: Insulin sensitivity decreased in subjects with the Thr54 allele of the FABP2 polymorphism when SFAs were replaced by MUFAs and carbohydrates.

KEY WORDS Ala54Thr polymorphism, steady state plasma glucose, Mediterranean diet, insulin sensitivity, dietary fat

INTRODUCTION
Insulin resistance is considered a risk factor for both diabetes and coronary heart disease, and it is measured by using the interaction between genetic and environmental factors. In general, high intakes of dietary fat have been associated with obesity and its comorbid conditions, including heart disease and type 2 diabetes. In most cases, the clinical expression of the disease can be prevented by dietary and lifestyle modifications (1). On the other hand, the genetic base of type 2 diabetes is very heterogeneous, and the disease has been related to various mutations in different genes that codify proteins linked to glucose and insulin metabolism, of which the insulin receptor (2), the insulin receptor substrate 1 (3), the Rad protein (4), glycogen synthase (5), and the β3-adrenergic receptor (6) are the best known. However, the genetic background of insulin resistance and type 2 diabetes is more complex and can also involve other genes that seemingly are unrelated to carbohydrate metabolism.

The fatty acid (FA)–binding protein 2 (FABP2) gene codes for intestinal FABP (IFABP), which is a member of a family of small (14–15-kDa) intracellular lipid-binding proteins. The gene located at 4q28-q31 has the conserved 4 exons and 3 introns that are characteristic of this family of genes (7, 8). IFABP plays important roles in several steps of fat absorption and transport: the uptake and trafficking of saturated and unsaturated long-chain fatty acids (LCFAs), the targeting of free FAs (FFAs) toward different metabolic pathways, protection of the cytosol from the cytotoxic effects of FFAs, and modulation of the enzyme additive involved in lipid metabolism (9, 10). Besides FFAs, IFABP may bind other ligands such as phenolic antioxidants, and it is abundant in the enterocyte, representing 2–3% of the cytoplasmic mass of those cells (11). It has been found that the expression of IFABP mRNA is under dietary control (12).

In 1995, Baier et al (13) reported a new G/A mutation. A transition (G→A) at codon 54 of FABP2 results in an amino acid substitution (Ala54→Thr54). This polymorphism is very

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common, with a Thr54 allelic frequency of ≈29% in most populations. This amino acid substitution was found to be associated with high fasting insulin concentrations, high insulin resistance, and high FA binding in Pima Indians (13).

In the current study, we investigated the possible influence of Thr54 polymorphism in the FABP2 gene on insulin sensitivity in a healthy young population. In addition, we investigated the interaction between this polymorphism and diet for insulin sensitivity.

SUBJECTS AND METHODS

Subjects and diets

Fifty-nine healthy normolipidemic subjects (total plasma cholesterol concentration: <5.2 mmol/L) who were attending the University of Cordoba volunteered to participate in the study. Of these subjects, 28 were homozygous for the most common Ala54 allele, and 31 were carriers of the Thr54 allele (Ala54/Thr54, n = 27; Thr54/Thr54, n = 4). All subjects underwent a comprehensive medical history, physical examination, and clinical chemistry analysis before enrollment. Subjects were <30 y old (± SD age: 21 ± 2 y) and had no evidence of any chronic illness such as hepatic, renal, thyroid, or cardiac dysfunction and no unusually high physical activity values. Mean initial body mass index (in kg/m²) was 21.4 ± 0.7 for Ala54 homozygotes, 21.7 ± 0.5 for Ala54/Thr54 heterozygotes, and 21.1 ± 0.5 for Thr54 homozygotes. This measure remained constant at the end of each diet.

Dietary information, including alcohol consumption, was collected over 7 consecutive days. Individual energy requirements were calculated by taking into consideration each subject’s weight and physical activity level. The subjects were encouraged to maintain their regular physical activity and lifestyle and were asked to record in a diary any event that could affect the outcome of the study, such as a stressful experience, a change in smoking habits, and consumption of alcohol or foods not included in the experimental design.

The study design included an initial 28-d period during which all subjects consumed a saturated FA (SFA)–enriched diet with 15% of energy as protein, 47% of energy as carbohydrates, and 38% of energy as fat (20% SFAs, 12% monounsaturated FAs (MUFAs), and 6% polyunsaturated FAs (PUFAs)). All subjects were then randomly assigned in a crossover design to 2 new dietary periods: a low-fat, high-carbohydrate diet (CHO diet) and a high-MUFA diet enriched with olive oil (Mediterranean diet). The 2 groups of subjects were assigned in random sequence order to 1 of the 2 dietary regimens for 28 d each. Group 1 (29 subjects) began with the Mediterranean diet and then switched to the CHO diet. In group 2 (30 subjects), the order of diets was reversed. The CHO diet (14) contained 15% of energy as protein, 57% of energy as carbohydrate, and 28% of energy as fat (<10% SFAs, 12% MUFAs, and 6% PUFAs). The Mediterranean diet contained 15% of energy as protein, 47% of energy as carbohydrate, and 38% of energy as fat (<10% SFAs, 22% MUFAs, and 6% PUFAs). Virgin olive oil provided 75% of total MUFAs consumed during the Mediterranean diet period. Each dietary period lasted 28 d. Dietary cholesterol was kept constant in our experimental design, and the mean cholesterol intake was 115 mg/1000 kcal during the 3 periods.

Written informed consent was obtained from all participants. The Human Investigation Review Committee at the Reina Sofia University Hospital approved this study.

The composition of the experimental diets was calculated by using the US Department of Agriculture food tables or the Spanish food-composition tables for local foodstuffs. Fourteen menus, prepared with regular solid foods, were rotated during the experimental period. We used virgin olive oil for cooking and salad dressing during the Mediterranean diet and palm oil and butter during the high-SFA diet. During the CHO diet, biscuits, bread, and jam replaced some olive oil or palm oil. Lunch and dinner were consumed in the hospital dining room, and breakfast and an afternoon coffee break were eaten in the medical school cafeteria. A dietitian supervised all meals. Duplicate samples from each menu were collected, homogenized, and stored at −80 °C. The protein, fat, and carbohydrate contents of the diet were analyzed by using standard methods (15). Evaluation of dietary compliance was also performed by examining the food diaries and by analyzing the FA content of the cholesterol ester fraction in LDL (16).

Blood sampling and biochemical determinations

Venous blood for insulin, glucose, lipid and lipoprotein analysis was collected from the subjects into EDTA-containing tubes after a 12-h overnight fast at the end of each dietary period. Each analysis was performed 3 times. Total cholesterol and triacylglycerols were assayed by enzymatic procedures (17, 18). HDL cholesterol was measured by analyzing the supernatant obtained after precipitation of a plasma aliquot with dextran sulfate-Mg+++ (19). LDL-cholesterol concentrations were calculated from the total and HDL-cholesterol and triacylglycerol values by using the formula of Friedewald et al (20). Unesterified free FA concentrations were measured by using an enzymatic colorimetric assay (Boehringer Mannheim, Mannheim, Germany) as described by Shimizu et al (21). To reduce interassay variation, plasma for biochemical determinations was stored at −80 °C and analyzed in duplicate at the end of the study.

Glucose suppression test

At the end of each dietary period, all subjects underwent a modified insulin suppression test (22, 23). Somatostatin (214 nmol/h), insulin (180 pmol · m⁻² · min⁻¹), and glucose (13.2 mol · m⁻² · min⁻¹) were infused into the same vein at 0800 after a 12-h fast. Somatostatin was used to inhibit endogenous insulin secretion. Blood was sampled every 30 min for the first 2.5 h, by which time steady state plasma glucose (SSPG) and steady state plasma insulin (SSI) concentrations were achieved. Blood was then sampled at 10-min intervals for the last 30 min (ie, at 150, 160, 170, and 180 min) for measurement of plasma glucose and insulin concentrations. These 4 values determined the SSPG and SSI concentrations. Because SSI concentrations were similar in all subjects, SSPG concentrations provided a measure of the ability of insulin to promote the disposal of infused glucose. Subjects with high SSPG are significantly more insulin resistant than those with lower SSPG.

Genotyping of FABP2 gene polymorphism

A polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) assay was developed for genotyping. The genomic DNA fragment flanking the Ala54/Thr54 polymorphism was amplified by using 2 primers flanking exon 2
of the FABP2 gene. The PCR was carried out with 250 ng genomic DNA and 0.2 μmol of each oligonucleotide primer (primer 1: 5’-CTACCGAGTTTCTTCCCCACC-3’; primer 2: 5’-AATTAACCCTCACTATAAGGC-3’) in a 50-μL final volume. DNA was denatured at 94 °C for 5 min; this was followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, and it concluded with a final extension at 72 °C for 10 min. The PCR product (10 μL) was digested with 5 units of restriction enzyme HhaI (New England Biolabs Inc, Beverly MA) in a total volume of 35 μL. The digested PCR products were resolved on 2% agarose gels. HhaI digested the wild type, alanine (GCT), which yielded 2 bands. Analysis of the cholesterol ester fraction of plasma LDL showed good adherence to the different diets (Table 2). During consumption of the SFA diet, there was a significantly higher concentration of palmitic acid (16:0) than that recorded during consumption of the CHO and Mediterranean diets (P < 0.05 for both). We also observed a significant increase in the concentrations of oleic acid (18:1) when subjects switched from the CHO diet to the Mediterranean diet (P < 0.05).

The baseline characteristics of the subjects according to the FABP2 Ala54/Thr54 polymorphism are shown in Table 3. There were no significant differences between subjects in any of the lipid variables after the 3 dietary periods.

SSPG and SSPG concentrations for the 3 genotypes of FABP2 Ala54/Thr54 polymorphism in response to each diet are shown in Table 4. Carriers of the Thr54 allele showed significantly (P < 0.05) higher concentrations of SSPG after an SFA diet than after a CHO diet.

### TABLE 1

<table>
<thead>
<tr>
<th>Protein (% of energy intake)</th>
<th>SFA diet</th>
<th>CHO diet</th>
<th>Mediterranean diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Analyzed 18.1 ± 2.5</td>
<td>17.6 ± 1.5</td>
<td>17.5 ± 2.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fat (% of energy intake)</th>
<th>Saturated</th>
<th>Monounsaturated</th>
<th>Polysaturated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated</td>
<td>20</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Analyzed 22.6 ± 4.1</td>
<td>9.2 ± 3.5</td>
<td>13.5 ± 1.2</td>
<td>5.0 ± 1.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carbohydrates (% of energy intake)</th>
<th>SFA diet</th>
<th>CHO diet</th>
<th>Mediterranean diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated</td>
<td>47</td>
<td>57</td>
<td>47</td>
</tr>
<tr>
<td>Analyzed 44.2 ± 8.3</td>
<td>54.5 ± 8.6</td>
<td>44.1 ± 7.8</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cholesterol (mg/dL)</th>
<th>SFA diet</th>
<th>CHO diet</th>
<th>Mediterranean diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated</td>
<td>115</td>
<td>115</td>
<td>115</td>
</tr>
<tr>
<td>Analyzed 112 ± 39</td>
<td>113 ± 48</td>
<td>117 ± 42</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Energy (MJ)</th>
<th>SFA diet</th>
<th>CHO diet</th>
<th>Mediterranean diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated</td>
<td>10.2</td>
<td>10.2</td>
<td>10.2</td>
</tr>
<tr>
<td>Analyzed 10.8 ± 1.1</td>
<td>10.6 ± 1.0</td>
<td>10.8 ± 1.5</td>
<td></td>
</tr>
</tbody>
</table>

1 All values are ± SD. SFA diet, saturated fatty acid–enriched diet; CHO diet, low-fat and high-carbohydrate diet; Mediterranean diet, high–monounsaturated fatty acid diet.

2 ± SD (all such values).

### Statistical analysis

Statistical analyses were carried out by using SPSS statistical software (version 11.0; SPSS Inc, Chicago, IL). Repeated-measures analysis of variance was used to analyze the effect of differences in plasma lipid, glucose, and SSPG concentrations between dietary phases. The general linear model for repeated-measures procedures was used to test the main effects of genes and diet and gene × diet interactions. When significant effects were observed, Tukey’s post hoc test was used to identify between-group differences. Because the Thr54/Thr54 group was small, the statistical analysis was performed for the combined Ala54/Thr54 and Thr54/Thr54 groups. Correlation was performed with Pearson’s correlation coefficient. FABP2 genotypes were dichotomized for these analyses. A P value of < 0.05 was considered significant. All data are given in the text and tables as means ± SD.

### RESULTS

Dietary composition was analyzed in duplicate meal portions, and results are shown in Table 1. The results were in good agreement with values obtained from the food-composition tables. Analysis of the cholesterol ester fraction of plasma LDL showed good adherence to the different diets (Table 2). During consumption of the SFA diet, there was a significantly higher concentration of palmitic acid (16:0) than that recorded during consumption of the CHO and Mediterranean diets (P < 0.05 for both). We also observed a significant increase in the concentrations of oleic acid (18:1) when subjects switched from the CHO diet to the Mediterranean diet (P < 0.05).

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### TABLE 3

<p>| Fatty acid composition of plasma LDL-cholesterol esters during each diet.1 |
|-------------------------------|-------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>SFA diet</th>
<th>CHO diet</th>
<th>Mediterranean diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>27.2 ± 1.4</td>
<td>18.9 ± 3.9</td>
<td>15.1 ± 0.4</td>
</tr>
<tr>
<td>16:1</td>
<td>2.2 ± 0.9</td>
<td>2.3 ± 0.3</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>18:0</td>
<td>2.8 ± 1.1</td>
<td>2.4 ± 0.8</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>18:1</td>
<td>45.5 ± 4.4b</td>
<td>38.5 ± 9b</td>
<td>49.7 ± 4.7b</td>
</tr>
<tr>
<td>18:2</td>
<td>20.2 ± 3.6a</td>
<td>33.6 ± 16b</td>
<td>26.4 ± 4.8b</td>
</tr>
</tbody>
</table>

1 All values are ± SD. SFA diet, saturated fatty acid–enriched diet; CHO diet, low-fat and high-carbohydrate diet; Mediterranean diet, high–monounsaturated fatty acid diet. Means in a row with different superscript letters are significantly different, P < 0.05 (repeated-measures ANOVA and Tukey’s test).
TABLE 4
Steady state plasma insulin and glucose (SSPI and SSPG) values for each genotype in response to each diet.

<table>
<thead>
<tr>
<th>Genotype and diet</th>
<th>SSPI pmol/L</th>
<th>SSPG mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala54/Ala54 (n = 28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFA diet</td>
<td>97.3 ± 22.8</td>
<td>6.4 ± 2.8</td>
</tr>
<tr>
<td>CHO diet</td>
<td>100.4 ± 23.9</td>
<td>5.6 ± 2.4</td>
</tr>
<tr>
<td>Mediterranean diet</td>
<td>99.2 ± 23.5</td>
<td>5.5 ± 2.8</td>
</tr>
<tr>
<td>Ala54/Thr54 (n = 27)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFA diet</td>
<td>99.3 ± 25.3</td>
<td>7.8 ± 4.2</td>
</tr>
<tr>
<td>CHO diet</td>
<td>97.5 ± 21.8</td>
<td>6.7 ± 3.1</td>
</tr>
<tr>
<td>Mediterranean diet</td>
<td>99.7 ± 25.3</td>
<td>6.1 ± 2.5</td>
</tr>
<tr>
<td>Thr54/Thr54 (n = 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFA diet</td>
<td>98.6 ± 30.7</td>
<td>7.4 ± 3.3</td>
</tr>
<tr>
<td>CHO diet</td>
<td>99.2 ± 23.5</td>
<td>5.0 ± 1.3</td>
</tr>
<tr>
<td>Mediterranean diet</td>
<td>97.2 ± 30.5</td>
<td>6.6 ± 0.9</td>
</tr>
</tbody>
</table>

* All values are x ± SD. SFA diet, saturated fatty acid–enriched diet; CHO diet, low-fat and high-carbohydrate diet; Ala54, alanine-encoding allele in codon 54 of the intestinal fatty acid–binding protein 2 gene; Thr54, threonine-encoding allele. Because the Thr54/Thr54 group was small, the statistical analysis was performed for the combined Ala54/Thr54+Thr54/Thr54 groups and is reported only on the Ala54/Thr54 means. Means in a column with different superscript letters are significantly different, P < 0.05 (repeated-measures ANOVA and Tukey’s test).

DISCUSSION

Our study shows that carriers of the Thr54 allele have less peripheral insulin sensitivity after the intake of an SFA diet than after that of an olive oil–rich, Mediterranean-type diet. Likewise, these subjects show an increase in FFA concentrations after consumption of an SFA diet than after that of a Mediterranean or CHO diet. However, in Ala54 allele homozygotes, no significant differences in SSPG concentrations were observed after any of the diets. These 2 facts together would indicate a genotype × diet interaction.

Reductions in SFA intake improve insulin sensitivity in patients with type 2 diabetes (24, 25), and these data agree with the results in healthy subjects in the current study. However, debate continues as to whether dietary SFA should be replaced with low fat and high carbohydrate or with MUFA. A study carried out in our laboratory showed that the CHO and Mediterranean diets are equally effective at promoting in vivo disposal of glucose, because they showed a similar decrease in SSPG (26). It is important to stress that the 3 diets in the current study provided the same fiber content. In addition, previous studies indicated that an increase in carbohydrates, without an increase in fiber, does not improve glucose metabolism (27). A study showed that diets with different carbohydrate content, but with an equal fiber content, improved glucose metabolism (28).

There is substantial evidence that lifestyle factors such physical activity and healthy diets reduce the risk of insulin resistance or type 2 diabetes. However, individual variability exists and is determined by genetic factors that influence tissue sensitivity to insulin.

Earlier studies carried out in Pima Indians by Baier et al (13) showed that carriers of the Thr54 allele presented increased incidence of hyperinsulinemia and insulin resistance after an oral glucose overload, in addition to a higher fasting lipid oxidation rate. These findings were consistent with the hypothesis that the presence of the Thr54 allele was associated with increased binding affinity to LCFAs, which resulted in enhanced intestinal absorption of FAs, higher plasma lipid concentrations, and, consequently, enhanced lipid oxidation rates, which would inhibit tissue sensitivity to insulin in vivo. It was later confirmed, in a healthy white population with normal glucose tolerance, that the Thr54 allele was associated with insulin resistance. These data coincide with those observed in the current study, but we noted the different peripheral insulin sensitivity in the Thr54 allele carriers only when the subjects consumed a diet rich in saturated fat, which suggested that an interaction between this polymorphism and the type of fat determined peripheral insulin sensitivity.

Previous studies showed that the FABP2 gene is a candidate gene that may be implicated in insulin resistance because its product is involved in FFA absorption and because defects in FFA regulation have been hypothesized to play a role in insulin resistance (29). IFABP expression appears to be limited to the enterocytes of the small intestine, where it is extremely abundant. The restricted expression of this protein combined with its high affinity for both unsaturated and saturated LCFAs indicates that IFABP has a role in the absorption and intracellular transport of dietary LCFAs. With this in mind, Baier et al (13) hypothesized that...
that the Ala54/Thr54 polymorphism results in increased FA uptake from the intestinal lumen. This hypothesis was based on in vitro findings that this polymorphism binds FAs with twice as much affinity as does the wild-type protein. Increased digestive absorption of fat could result in abnormally high postprandial plasma FA concentrations, which produce transient muscle cell insulin resistance via the glucose-FA cycle, and in high hepatic glucose production via increased gluconeogenesis. In the current study, we observed that subjects with the Thr54 allele had higher FFA concentrations than did those who were homozygous for the Ala54 allele when consuming the SFA diet. Considering the hypothesis proposed by Baier et al (13), these results suggested a plausible mechanism of the FABP2 Ala54/Thr54 polymorphism × diet interaction for determining insulin sensitivity.

Thus, the presence of the FABP2 gene Ala54/Thr54 polymorphism impairs peripheral insulin sensitivity when the carriers consume an SFA diet.

We express our sincere thanks to the study participants.

REFERENCES


Are the psychological tests valid?

Dear Sir:

The article by Black et al (1) provides insufficient information to claim that weekly administration of iron and zinc supplements benefits exploratory behavior. The iron and the iron-plus-zinc treatments had a significant effect on the orientation-engagement factor of the Bayley Behavior Rating Scale (2), which includes one item (out of 11) on exploration. This factor also includes items that assess “… arousal, positive affect, energy, initiative, enthusiasm, exploration, social engagement, and lack of fearfulness” (3). At issue is not a simple change of labels but whether the 11 items included in the orientation-engagement factor measure the concept of exploration. No evidence to this effect was presented, and the statement that “Orientation-engagement factor served as the measurement of exploration” trivializes both the scale and the very nature of construct validity (4). The definition of exploration should not be left to common sense; it requires careful consideration of the behavioral and developmental components of the concept.

There was no treatment effect on the Mental Development Index (MDI) from the Bayley Infant Development Scale II administered at 12 mo. This finding was not surprising. The MDI obtained at 12 mo has a track record of poor sensitivity to detect developmental delays secondary to micronutrient deficiencies, and its construct validity is questionable (5, 6). Accordingly, the authors could have predicted that the MDI would not discriminate among groups after treatment. The probabilities of detecting effects on the mental scale, if any were indeed present, would have increased if the authors had charted a developmental trajectory after age 12 mo (6, 7).

The author had no conflicts of interest.

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REFERENCES


Reply to E Pollitt

Dear Sir:

We thank Pollitt for his interest and comments regarding our investigation of the effect of micronutrient supplementation on children’s development (1).

The first comment made by Pollitt concerned our use of the term “exploration.” Exploration refers to infants’ use of their senses, motivation, and emerging motor and mental skills to learn about their physical and social environment. Exploration and child development are thought to be interactive, bidirectional processes; exploration enriches infants’ developmental skills, and, as infants’ mental and motor skills mature, they are capable of more sophisticated exploration.

In our investigation of micronutrient supplementation, we were particularly interested in exploration because it plays an important role in the theory of functional isolation (2), which serves as a possible explanation for the association between nutritional deficiency and delays in children’s development. Infants with low rates of exploration may miss opportunities for the physical and social enrichment that advance their developmental skills. If micronutrient supplementation promotes exploration, as we found in our recent investigation (1), it may be an important mechanism in understanding associations between micronutrient deficiency and delays in early child development.

Exploration is often assessed through the observation of infants during play. In our investigation, we observed infants during a warm-up period and during the administration of the Bayley Scales of Infant Development, II (3). We used the “orientation-engagement” factor of the Behavior Rating Scale of the Bayley Scales as an operational definition of exploration because it measures “the child’s proclivities toward approaching or avoiding environmental interactions that are task-related or social in nature” (3). For 6–12-mo-old infants, the orientation-engagement factor includes 11 behaviors: social engagement, enthusiasm, persistence in completing tasks, exploration, initiative, interest in materials, energy, positive affect, lack of fearfulness, state of arousal, and stability of state of arousal. Each behavior is assessed by a trained examiner using a 5-point Likert scale after administration of the mental and motor scales of the Bayley Scales. In keeping with the psychometric properties reported for
the standardization sample (3) in our investigation, the internal consistency of the orientation-engagement factor exceeded 0.87 during both observations. High scores represent endorsement of the behaviors related to the factor. Thus, a 6–12-mo-old infant with a high score in the orientation-engagement factor was observed to be alert, to be enthusiastic, to be persistent, and to have initiated interactions with materials and people in the testing setting—behaviors that are consistent with exploration in the second 6 mo of life.

The second comment by Pollitt involved the use of the Mental Developmental Index (MDI) of the Bayley Scales of Infant Development, II to examine changes in mental development related to micronutrient supplementation. The MDI represents a complex integration of empirically derived cognitive skills that are based on maturation and other theories of infant development. Although the MDI is probably the most well-standardized, widely used assessment of infant mental development in the world, evidence substantiates the low predictive validity of infant assessments of mental development, such as the MDI, for infants younger than 24 mo to subsequent measures of intelligence (4, 5). The lack of continuity may be partially explained by the multidimensional and rapidly changing aspects of infant mental development and by variations in performance during infancy, variations in tasks used to measure intellectual functioning throughout childhood, and variations in environmental challenges and opportunities that may influence development. Predictability appears to be better when investigators focus on specific cognitive, motivational, or behavioral processes (6).

One might ask why the MDI is so widely used to investigate associations between nutritional supplements and mental development despite its limited predictability. The reasons are many. First, the MDI is a well-standardized, psychometrically strong measure of infant mental development. Because it is an age-normed test, MDI scores can be used to compare the performance of children with that of same-age peers across ages, cultures, and conditions from birth through 42 mo of age. Second, MDI scores are sensitive to deviations in early development associated with environmental and nutritional conditions, such as low birth weight (7). For example, changes in MDI have been reported in response to both iron (8) and zinc (9) supplementation in infants younger than 18 mo. Third, predictability appears to be better among infants with early medical or environmental challenges, such as nutritional deprivation, than among healthy infants (10). Finally, because there is no consensus regarding the mechanisms linking micronutrient deficiency and child development (11), it is not clear what aspects of mental development should be investigated. One alternative is to combine a well-standardized assessment of mental development, such as the MDI, with measures of specific processes thought to be sensitive to the nutritional deficiencies under investigation.

We agree with Pollitt’s recommendation to examine how developmental trajectories are related to children’s nutrition. As we have shown among children with failure-to-thrive, cognitive development is optimally examined through pathways that begin in the first year of life, extend through at least early school age, and focus on the integration and organization of biological, nutritional, and psychosocial challenges and opportunities (12). Our investigation of changes in motor, mental, and behavioral development from 6 to 12 mo of age related to micronutrient supplementation (1) is a step in that process.

Neither author had a conflict of interest.

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REFERENCES
Erratum


On page 1604, paragraph 3 of the abstract, the age of the children mentioned (28–60 mo) is inaccurate. The sentence should read, “The study involved the longitudinal follow-up of children aged 0–95 mo for clinical malaria episodes and anthropometric measurements through 4 cross-sectional surveys.”

Erratum


The legend printed with Figure 1 of this article was not complete. The figure and the complete legend appear below.

FIGURE 1. Plot of log10(thyroid volume (Tvol)) and log10(urinary iodine (UI) concentration) showing a Lowess smoothed line calculated for an international sample of 6–12-y-old children (n = 3319), with sexes and sites combined. Log (Tvol) begins to increase at log (UI) >2.7 (dotted line), which, transformed back to the linear scale, corresponds to a UI concentration of ≈500 μg/L.
The first chapter of this book covers more than everything you ever wanted to know about the structures, synthesis, and biosynthesis of vitamin E. The next chapter provides the human health implications related to vitamin E and a brief review of the highlights of the new dietary reference intakes for vitamin E developed by the Food and Nutrition Board (1). As a result of the publication of the dietary reference intakes for vitamin E, there is increased awareness in the nutrition community that not all forms of vitamin E are the same and that humans specifically require α-tocopherol, a form that is limiting in the diet. Unfortunately, for decades, the contents of vitamin E in foods have been reported in units of α-tocopherol equivalents (ATE). ATEs blur the distinction between vitamin E–rich foods and α-tocopherol–rich foods. For example, it is shown in Table 8.6 that soybean oil (per 100 g) contains 107 mg ATE but only 8 mg α-tocopherol. Chapter 8 provides a listing of the amounts of the various forms of vitamin E in a goodly selection of foods and provides values from different sources, which allows the reader some indication of the variability in measurements. This listing also emphasizes the relative paucity of vitamin E in fruit and vegetables.

Not only are the methods for vitamin E analysis well described and compared in this book, but other factors that influence the dietary content of vitamin E are covered in chapters that focus on vitamin E as a food antioxidant. A rather interesting chapter covers research that has been conducted to investigate the effects of vitamin E supplementation in cows, sheep, pigs, and chicken for the purpose of improving the quality of meat, milk, and eggs. Another “hot” topic that is covered is the stability of vitamin E during food processing, preparation, and storage and how these factors reduce the vitamin E content of some foods. These are important factors given the labile nature of vitamin E and the controversy over how much dietary vitamin E is actually consumed.

My favorite chapter is the one that covers the prooxidant effects of vitamin E. One of the more interesting debates in the vitamin E community concerns the in vivo role of α-tocopherol as a prooxidant when coantioxidants are limiting. Oil chemists have been studying these phenomena for decades. Strikingly, as discussed in chapter 3, α-tocopherol, but not γ-tocopherol, becomes a prooxidant at high concentrations (>250 ppm) in oils. Such α-tocopherol concentrations are >10 times those found in human plasma or tissues, which suggests that the body limits α-tocopherol accumulation. However, this potent antioxidant phenomenon does not explain why γ-tocopherol concentrations in the body are one-tenth those of α-tocopherol. However, the antioxidant power of γ-tocopherol is clearly beneficial to plants exposed to sunlight and damaging ultraviolet irradiation and is why plants preferentially synthesize high γ-tocopherol–containing oils. Eitenmiller and Lee are to be congratulated for their interesting and well-written book, which contains an amazingly extensive list of reference citations (>1000).

Maret G Traber

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REFERENCE


This book, a result of a Nestle Nutrition Workshop on micronutrient deficiencies in the first 6 mo of life, contains 16 chapters written by experts in their fields. The first 3 chapters provide excellent background overviews, including up-to-date summary tables. Chapter 1 summarizes biochemical tests for assessing the micronutrient status of both infants and mothers. Chapter 2 highlights uncertainties surrounding the dietary reference intakes of young infants and future research needs; examples for 3 micronutrients of special public health significance in developing countries—vitamin A, iron, and zinc—are provided. Chapter 3 addresses the micronutrient adequacy of the diets of young infants. The paucity of data on the extent to which undernourished women can supply their exclusively breastfed infants with the recommended amounts of the high risk micronutrients, namely, thiamine, riboflavin, selenium and vitamins A, B-6, B-12, and C is highlighted. This adequacy assessment considers both the micronutrient content of breast milk and the volume consumed and involves a discussion of the possible strategies for increasing the delivery of these micronutrients.

Five chapters deal with the etiology, health consequences, and prevention of individual deficiencies of iodine, vitamin A, zinc, vitamin K, or vitamin D during early infancy. A chapter on selenium and vitamin E focuses on host defense and resistance to infection. A chapter on iron emphasizes the relation between maternal deficiency and infant health outcomes, specifically birth weight, prematurity, anemia, and mortality. The multiple


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REFERENCE
causes of anemia are the focus of a separate chapter. Three other chapters cover the effects of multimicronutrient supplements either during periconception, when the emphasis is on reducing the risk of birth defects, or in later pregnancy, when infant outcomes amenable to prenatal multimicronutrient interventions are highlighted. These interventions can have both positive and negative effects; therefore, a cautionary note is included on the universal use of multimicronutrient supplements under circumstances in which the effects on public health are not well understood. The effects of iron or iodine deficiency during pregnancy and early infancy on mental and psychomotor development are described in another chapter.

Of special interest is a chapter that emphasizes the special micronutrient needs of preterm infants to ensure adequate enteral and parenteral intakes of iron, zinc, copper, selenium, chromium, iodine, and manganese. In addition, there is an important chapter on the role of micronutrient status in modulating fetal and child health in the presence of HIV infection, with emphasis on deficiencies of vitamin A, zinc, and selenium. Inconsistencies in the results of randomized controlled trials of micronutrients in relation to micronutrient supplementation during pregnancy and the risk of vertical transmission are highlighted, as are fetal outcomes such as low birth weight, prematurity, and fetal death; the need for more research is emphasized.

Although the chapters differ in style and approach, this book is a useful integrated resource that provides health professionals with an excellent update of an important and often overlooked area. Micronutrient deficiencies in young infants are difficult to identify because of their often subtle but sometimes irreversible health consequences, a point that is emphasized in the verbatim discussion that follows each chapter.

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This book is one of a series reporting a conference held in Bangkok, Thailand, in November 2003. It consists of an uneven mixture of articles covering a broad range of nutrition topics. These include information and guidelines on the nutrition of general populations, such as over- and undernutrition as global problems; minerals and trace elements in disease; and specific overviews of clinical syndromes, from diagnosis and definition through pathophysiology and treatment. Despite the location of the conference, however, none of the articles focus on nutrition-related issues in specific populations from Asia. Some chapters review inflammatory cytokines in obesity, with comments on insulin resistance and diabetes; the metabolic syndrome as a clinical problem; and fatty acid isomers in lipid metabolism. Although a few chapters are quite detailed and focused, most are brief overviews meant to introduce a topic to a relatively naive reader. Carbohydrate, lipid, and protein metabolism are all covered generally with an admirable focus on human and clinical aspects; there are also several topics related to fluid and electrolyte balance and their relation to disease.

The total volume is only 272 pages and therefore this is but a snapshot of the thinking of some of the prominent experts in these fields. Because the meeting took place in 2003, few references are more recent than 2001–2002, which means that the report will be primarily useful to a reader who desires a broad overview of clinical nutrition at that time rather than a detailed account. A quick review of chapter headings can help the reader determine which topics are included.

Meeting reports such as this are usually most useful to workers in the field and as a way to disseminate to others with similar interests what was presented and discussed at the meeting. This volume follows that pattern and contains the usual mix of straightforward reviews of the authors’ areas of interest with some presentations of controversial new ideas. As a result, the volume is uneven, with 16 different authors and no overriding theme. Instead, the book presents reports that are related distantly, if at all, and contains material requiring different levels of reader expertise for comprehension. In general, however, the writing is clear and understandable, with reasonable and frequent summary diagrams. As meeting reports go, this is a worthy contribution.

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Science-based solutions to obesity: what are the roles of academia, government, industry, and health care?1–5

George L Blackburn and W Allan Walker

ABSTRACT
The complexity of the obesity epidemic requires the cooperation of key stakeholders in this effort. No one sector, academia, government, industry, or health care, has been successful in combating this disease to date. On March 10–11, 2004, The Harvard Medical School Division of Nutrition hosted the symposium “Science-Based Solutions to Obesity: What is the Role of Academia, Government, Industry, and Health Care?” as a platform to address the role of these stakeholders, both individually and collectively, in combating the nation’s epidemic of obesity. The proceedings from the symposium, included in this supplement, discuss the following: the science of obesity-related topics such as genetics, protein and weight loss, portion size, energy density, and behavior; the need for more aggressive government policies; industry’s role in using research and development capabilities to promote healthier, portion-controlled products; and how to translate nutrition information from medical doctors to patients. Am J Clin Nutr 2005;82(suppl):207S–10S.

KEY WORDS Health care, nutrition, obesity, overweight, symposium

Obesity is one of the most daunting health challenges of the 21st century (1). It is related to ≈1–400 000 deaths per year (2) and costs society an estimated $117 billion in direct and indirect costs (3). Between 1986 and 2000, the prevalence of severe obesity [body mass index (BMI) ≥40 kg/m2] quadrupled from 1 in 200 Americans to 1 in 50. Adults with a BMI of ≥50 kg/m2 (super-obese) increased by a factor of 5, from 1 in 2000 to 1 in 400 (4, 5). Children and adolescents suffered a similar fate. In the past 30 y, the prevalence of overweight in pediatric age groups has nearly tripled (6). At present, ≈9 million children over 6 y of age are considered obese (7).

The symposium’s theme of collaboration mirrored the direction of national health policy in the fight against obesity. The National Institutes of Health (NIH) has launched a cohesive, multidimensional, NIH-wide research agenda for addressing the problem of obesity. In 2003, the National Heart, Lung, and Blood Institute held a “Think Tank,” a major meeting that brought together a diverse group of stakeholders from the academic, consumer, and professional communities to develop research recommendations (1). The symposium brought these groups together again to assess progress in the science, treatment, and prevention of obesity.

America’s losing battle against obesity has made it clear that teamwork, the best combined efforts of academia, government, industry, and health care, is required to stop the epidemic. Each member of the team has a critical role to play. The symposium provided a forum in which to explore those roles. This year’s event included 14 presentations, two general discussions, and a panel session with representatives from academia, government, industry, and health care. Speakers covered a wide range of topics encompassing different disciplines of ongoing obesity research. The first day focused on the science and physiology of obesity and weight control; the second focused on strategic initiatives to address the problem.

A presentation by Allen M Spiegel (8) described the NIH Obesity Research Task Force. Established in 2003, its charge is to engage NIH-wide resources in a coordinated campaign to accelerate progress in obesity research. The Task Force recently released its Strategic Plan for NIH Obesity Research (1), an ambitious and detailed agenda that leverages expertise throughout the NIH and targets research efforts to the areas of greatest promise, including molecular, physiologic, and behavioral research.

Another session focused on the relationship between obesity and genes (9). Evidence from twin and adoption studies indicates that genes make substantial contributions to obesity (10). Twin

1 From the Harvard Medical School Division of Nutrition, Boston, MA.
3 This symposium was supported by The Coca-Cola Company Family of Brands, ConAgra Foods, Healthy Foods of America, McNeil Nutritional, Nestle Nutrition Institute, Nutrition & Health Partnership, The Peanut Institute, Slim Fast Foods Company, and Wyeth Nutritional. The proceedings of this symposium are published as a supplement to the American Journal of Clinical Nutrition. This supplement is a synthesis of the presentations, discussions, and ideas put forth over those two days. Guest editors for the supplement publication were George Blackburn (Harvard Medical School) and W Allan Walker (Harvard Medical School). The Harvard Medical School Division of Nutrition, in conjunction with the American Dietetic Association Foundation and the ADA Weight Management Practice Group, sponsored an additional presentation of relevant sections of this symposium at the ADA Food and Nutrition Conference Expo on October 2, 2004, in Anaheim, CA.
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studies in the context of overfeeding and energy-deficit experiments suggest that gene-environment interactions also affect energy balance (11). Joel Hirschhorn described methods being used to identify human obesity genes and noted the importance of animal models in advancing understanding.

George L. Blackburn (12) identified the need to update and refine medical school curricula as a key objective in the fight against obesity. He focused on widespread health illiteracy (13): how it perpetuates the epidemic and how best practices in teaching and learning can help doctors teach their patients about the basics of healthy eating. Evidence suggests that the process of change is a gradual one, especially in adults (14). Blackburn explained that The Academy at Harvard Medical School (15) has started that process by spearheading innovations in education, by giving physicians the knowledge (16) and skills they need to help patients make healthy foods choices (17).

In noting that physicians face a world of new challenges, best addressed by innovation, Blackburn cited changes in the highly regarded advisory report on the 2005 Dietary Guidelines for Americans (18, 19), particularly the high-profile use of Institute of Medicine reference intakes for macronutrients (20). He also described how the first of nine key Dietary Guidelines messages, consume a variety of foods within and among the basic food groups while staying within energy needs, mirrors the acronym CQE (Count Calories, Choose Quality Foods, and Exercise Daily), a concept developed by S. Daniel Abraham, founder of SlimFast Foods and the driving force behind the Center for Healthy Living at Harvard Medical School (21).

The Nutrition Academic Awards Program (22) is another effort to spearhead innovations, in nutrition education and in ways to integrate the subject into medical school curricula and medical practice. Nutrition is known to be an important component in establishing a healthy lifestyle and preventing major causes of diseases that commonly affect Americans (23). Any approach that helps physicians practice preventive medicine, and teach their patients how to do so, contributes to the national health agenda. W Allan Walker described two such approaches, the WAVE (weight, activity, variety, and excess) and REAP (rapid eating and activity assessment for patients) assessment tools, developed by a Nutrition Academic Awards recipient at Brown University (24, 25).

Another presentation discussed tools and tactics used by those who have been successful at long-term weight loss (26), those who have lost ≥30 lb (14 kg) and maintained the loss for more than 1 y. Data from the National Weight Control Registry (27) indicate that diet and exercise are the cornerstones of success. Rena Wing described patterns of behaviors associated with long-term weight loss, including the following: eating a low-fat, low-calorie diet; having breakfast; tracking weight on regular basis; eating a consistent diet; quickly addressing small weight gains; calorie diet; having breakfast; tracking weight on regular basis; eating a consistent diet; quickly addressing small weight gains; and maintaining high levels of physical activity.

In a session on Physical Activity and Body Weight Control, John Jakicic (28) described the role that physical activity plays in maintaining weight loss. Studies show that exercise is an important component of weight-control programs and that a higher duration and intensity of exercise may improve long-term weight loss (29). Jakicic explained that sedentary lifestyles and obesity are intricately linked (30) and encouraged health care providers to identify and address obstacles that prevent their patients from becoming more active (29).

Gary Foster (31) spoke on the subject of behavior, particularly the influence of health care providers on the behavior of their patients. Studies suggest that brief interventions by physicians can change patient behavior (32) and that frequent contact with health care providers can contribute to success. He noted that patients respond strongly to positive reinforcement and cited data showing that health care professionals can play a pivotal role in curbing the epidemics of obesity and inactivity (30).

The degree to which the obesogenic environment undermines that role is of growing interest to academia, government, industry, and health care. In a presentation on Portion Size and the Obesity Epidemic, Barbara Rolls (33) discussed the contribution of ever-bigger serving sizes to the obesity crisis and the connection between energy density and satiety. Data indicate that the larger the portion, the more people eat (34). They also show that low-energy, high-density foods (eg, fruits, vegetables, and salads) provide satiety that can result in decreased total intake (35).

Popular diets (eg, the Atkins Diet) have brought increased attention to macronutrient intake, especially protein. Recent studies suggest that high-protein diets produce greater short-term weight loss than low-fat diets (36) but that the difference does not last beyond 1 y (37–39). High-protein diets are also purported to improve cardiovascular risk factors and blood lipid profiles. In his presentation on Protein, Body Weight, and Cardiovascular Health, Frank Hu (40) reviewed recent research examining the effects of protein on cardiovascular health, and he underscored the need for long-term data on the safety of high-protein, ketogenic diets.

To date, weight loss surgery (41) is recognized as the most effective means for producing long-term weight loss and reduction of obesity-related comorbidities in appropriately selected patients (42–44). Of the various surgical options available, Roux-en-Y gastric bypass is considered the gold standard (44). Surgery is fundamentally different from dieting. It changes the physiology to reset energy equilibrium, affects the complex weight regulatory system at multiple levels, inhibits environmental influences on weight regulation, and defeats powerful mechanisms that are inappropriately active in obesity.

Ultimately, obesity is a matter of imbalance: a mismatch between energy in and energy out. Susan Finn (45) discussed the ways that industry is working to correct that imbalance. In particular, she cited community-based programs for children, commitments to develop and market healthy products that promote portion control, and a willingness to practice responsible advertising. Donald Short (46), in a presentation on Coca-Cola’s initiatives to fight obesity, underscored the importance of coordinated efforts by business, academia, government, and health care. Pat Verduin (47) from ConAgra echoed that message, noting that industry is already bringing healthier foods to market, that it has a critical role to play in public education, and that strong partnerships will benefit everyone.

Tomas Philipson (48) discussed the Food and Drug Administration’s multifaceted attack on obesity, a broadside that includes targeted messaging and proposed changes in the design of food labels. He cited the need to make food labels easier to read and understand and to improve communication with an increasingly diverse US population. Adam Drewnowski (49) addressed the related subject of health disparities in America. He cited statistics showing that the prevalence of overweight and obesity is higher in minority groups, especially African Americans.
and Hispanics, it is than in whites (1). He noted that the ultimate goal of the NIH is to give every American an equal opportunity to lead a healthy life. As long as the obesity epidemic goes unchecked, that goal will remain out of reach. Obesity will continue to take its toll on the health care system, the economy, and the quality of life for millions of Americans. A recent study estimated annual medical spending as a result of overweight and obesity (BMI ≥25) to be as much as $92.6 billion in 2002 dollars (9.1% of US health expenditures) (50). Annual indirect costs of obesity to US businesses in the form of health insurance expenditures, paid sick leave, life insurance, and disability insurance have been estimated at $12.7 billion (51–53).

At the final panel discussion of the 2004 Postgraduate Nutrition Symposium at Harvard Medical School, Science-Based Solutions to Obesity: What are the Roles of Academia, Government, Industry, and Health Care?, someone from the audience asked a question: Where do we go from here? The science and physiology of obesity, and the efforts underway to fight it, were the focus of this year’s symposium, illustrating that we are now attacking the problem on every possible front, from laboratories at major pharmaceutical firms to local community health centers. The diversity represented by the symposium’s guest speakers, and their commitment to a work together in common cause, is significant in and of itself, a milestone in the fight against obesity. In a closing statement, George L. Blackburn noted that we have come a long way, even in just the past 5 y, but still have a long way to go (54).

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Executive summary of the Strategic Plan for National Institutes of Health Obesity Research\textsuperscript{1–4}

Allen M Spiegel and Barbara M Alving

ABSTRACT
The Strategic Plan for National Institutes of Health (NIH) Obesity Research is intended to serve as a guide for coordinating obesity research activities across the NIH and for enhancing the development of new efforts based on identification of areas of greatest scientific opportunity and challenge. Developed by the NIH Obesity Research Task Force with critical input from external scientists and the public, the Strategic Plan reflects a dynamic planning process and presents a multidimensional research agenda, with an interrelated set of goals and strategies for achieving the goals. The major scientific themes around which the Strategic Plan is framed include the following: preventing and treating obesity through lifestyle modification; preventing and treating obesity through pharmacologic, surgical, or other medical approaches; breaking the link between obesity and its associated health conditions; and cross-cutting topics, including health disparities, technology, fostering of interdisciplinary research teams, investigator training, translational research, and education/outreach efforts. Through the efforts described in the Strategic Plan for NIH Obesity Research, the NIH will strive to facilitate and accelerate progress in obesity research to improve public health. \textit{Am J Clin Nutr} 2005;82(suppl):211S–4S.

KEY WORDS Obesity, research, National Institutes of Health, strategic plan, nutrition, diet, physical activity, behavior, environment, medical research, interdisciplinary research, translation, health disparities

INTRODUCTION
Obesity has risen to epidemic levels in the United States. It leads to devastating and costly health problems, reduces life expectancy, and is associated with stigma and discrimination. Obesity is a strong risk factor for such serious diseases as type 2 diabetes and heart disease; it is also a risk factor for certain cancers and is associated with depression and other medical conditions. More than 65\% of US adults are overweight or obese, with nearly 31\% of adults (more than 61 million people) meeting criteria for obesity. Furthermore, although obesity and overweight have risen in the population in general, the greatest increases observed over approximately the past two decades have been in the prevalence of extreme obesity; those who are severely obese are most at risk for serious health problems. Levels of childhood overweight have nearly tripled since 1970: \( \approx 16\% \) of children and teens ages 6 through 19 y are now overweight. The levels of pediatric overweight have ominous implications for the development of serious diseases, both during youth and later in adulthood. Overweight and obesity also disproportionately affect racial and ethnic minority populations and those of lower socioeconomic status. Left unabated, the escalating rates of obesity in the US population will place a severe burden on the nation’s health and its health care system.

OBESITY AND NATIONAL INSTITUTES OF HEALTH RESEARCH
On the surface, it may seem that the solution to the obesity epidemic is obvious: “get people to eat less and exercise more.” The reality is that this change is very difficult to accomplish, and research is critical to address the issue successfully. Given the complexity and multiplicity of the forces driving the obesity epidemic, the National Institutes of Health (NIH) recognizes that it cannot, by itself, solve this major public health problem. However, the NIH can and must be a key contributor to solving the obesity problem through scientific research. Through its research mission, the NIH is seeking to capitalize on recent scientific discoveries to propel new efforts toward further understanding the forces contributing to obesity and toward developing strategies for its prevention and treatment.

The increase in obesity has been fueled by a complex interplay of environmental, social, economic, and behavioral factors, acting on a background of genetic susceptibility. Thus, the NIH supports a broad spectrum of obesity-related research, including molecular, genetic, behavioral, environmental, clinical, and epidemiologic studies. The challenges of today’s obesity epidemic are daunting, yet the discoveries emanating from previous research investments offer unprecedented opportunities for new scientific research efforts to help meet these challenges.

\textsuperscript{1} From the National Institute of Diabetes and Digestive and Kidney Diseases (AMS) and the National Heart, Lung, and Blood Institute (BMA), National Institutes of Health, Bethesda, MD.


\textsuperscript{3} Reprints will be available on the NIH Obesity Research website (http://obesityresearch.nih.gov) as part of the entire Strategic Plan for NIH Obesity Research.

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THE STRATEGIC PLAN FOR NIH OBESITY RESEARCH: INFORMING THE PROCESS

Purpose and formulation of the Strategic Plan

The purpose of the Strategic Plan for NIH Obesity Research is to provide a guide for coordinating obesity research activities across the NIH and for enhancing the development of new research efforts based on identification of areas of greatest scientific opportunity and challenge. The Strategic Plan represents a cohesive, multidimensional research agenda for addressing the problem of obesity. It includes short-, intermediate-, and long-term goals for basic, clinical, and population-based obesity research, along with strategies for achieving those goals that also range in timeframe. Building on scientific advances from previous NIH-supported efforts, the Strategic Plan seeks to maximize collaboration among the NIH ICs and capitalize on their expertise and interest in developing obesity research initiatives.

Importantly, the planning process was informed by input from external experts through interactions with NIH staff at scientific meetings, through meetings and workshops convened by NIH ICs for the purpose of obtaining research planning advice, and through presentations by the cochairs of the Task Force to external scientific and health advocacy organizations. For example, a “Think Tank” meeting on enhancing obesity research, held by the National Heart, Lung, and Blood Institute, brought together a diverse group of stakeholders from the academic, consumer, and professional communities to provide research recommendations. Recent general scientific meetings offered a venue for NIH staff to glean external input and advice on obesity research planning. For example, a January 2003 Keystone meeting provided a means of tapping external scientific expertise regarding the pathogenesis and treatment of obesity. Later in 2003, the cochairs of the NIH Obesity Research Task Force gave the keynote lecture at the Annual Scientific Meeting of the North American Association for the Study of Obesity; this presentation afforded an opportunity to gain feedback from attendees on NIH obesity research efforts. An important advisory group from whom the NIH receives expert input on obesity is the National Institute of Diabetes and Digestive and Kidney Diseases Clinical Obesity Research Panel, which is composed of leading external obesity researchers and clinicians. Additionally, strategies designed to achieve the goals of the Strategic Plan, in the form of initiatives, are reviewed and discussed by members of the ICs’ National Advisory Councils, which are groups of prominent external scientific experts and lay leaders established by law and charter to provide advice to the ICs.

As with any future-oriented plan, the Strategic Plan for NIH Obesity Research is intended to be dynamic. As new scientific opportunities arise from current research investments and accomplishments, the research planning process will evolve to build on these areas, thus accelerating research in the most promising directions to continue to meet the challenges of obesity.

STRATEGIC PLAN FOR NIH OBESITY RESEARCH: GOALS FOR NIH OBESITY RESEARCH AND STRATEGIES FOR ACHIEVING THE GOALS

The Strategic Plan contains a cohesive set of interrelated goals for achievements in NIH obesity research. The goals are organized under the following four themes: 1) research toward preventing and treating obesity through lifestyle modification; 2) research toward preventing and treating obesity through pharmacologic, surgical, or other medical approaches; 3) research toward breaking the link between obesity and its associated health conditions; and 4) cross-cutting research topics, including health disparities, technology, fostering of multidisciplinary and
interdisciplinary research teams, investigator training, translational research, and education/outreach efforts.

For each of these four themes, goals for the short-, intermediate-, and long-term time horizons are presented, followed by a set of strategies for achieving the goals.

Research toward preventing and treating obesity through lifestyle modification

Under this theme, the goals and strategies for achieving them encompass identifying modifiable behavioral and environmental factors that contribute to the development of obesity in children and adults and designing and testing potential intervention strategies. Research will build on the results of clinical trials that demonstrated successful behavioral and environmental approaches to lifestyle modification. The effects of specific modifications in diet and physical activity will be studied, as will modifications of environmental factors that promote overconsumption of food and sedentary lifestyles. Two examples of such research are work-site interventions to prevent obesity and studies of the “built environment” and its relationship to physical activity.

Research toward preventing and treating obesity through pharmacologic, surgical, or other medical approaches

Under this theme, the goals and strategies for achieving them encompass the continued elucidation of the molecules and biological pathways that regulate appetite, energy expenditure, and the storage of energy as fat, and accelerating the design and testing of treatment and prevention strategies. Research will include additional genetic and other molecular studies. With respect to genetic research, some individuals are far more susceptible to developing obesity in a given environment than others. In rare cases of severe, early-onset obesity, this susceptibility results from a single genetic abnormality. However, more common forms of obesity are genetically complex, likely involving interactions of variations in multiple genes to increase susceptibility. The identification of genes involved in obesity will enhance efforts toward prevention and treatment. Through genetic studies and other molecular research, the NIH can identify potential new targets for drug development. These “targets” would be the molecules and pathways involved in regulating energy balance: the balance between energy intake (through feeding) and energy expenditure (through physical activity and maintaining basic body functioning). Thus, research in these areas will contribute to the development of medical strategies that affect energy balance to help prevent or treat obesity.

Research toward breaking the link between obesity and its associated health conditions

Under this theme, the goals and strategies for achieving the goals encompass building on research that illuminates the connection between obesity and type 2 diabetes, cardiovascular disease, cancer, and other diseases. Major recent advances in the understanding of fat cell metabolism include the appreciation that fat cells secrete hormones that promote inflammation and hypertension. Understanding the different mechanisms of various body fat depots in causing insulin resistance (a precursor to diabetes) and other metabolic abnormalities is a fundamental question that must be addressed. Such research efforts as the recent creation of a bariatric surgery clinical research consortium and the formation of a network to study nonalcoholic fatty liver disease (a major new cause of liver failure associated with the obesity epidemic) will help to identify the mechanisms linking obesity to other serious health conditions and will open the possibility of breaking the link between them.

Cross-cutting research topics, including health disparities, technology, fostering of multidisciplinary and interdisciplinary research teams and investigator training, translational research, and education/outreach efforts

The cross-cutting research theme encompasses several topics. First, of critical importance is a focus on the needs of specific populations, including children, racial/ethnic minorities who are disproportionately affected by obesity, persons living in conditions of lower socioeconomic status or who have low literacy, women, older adults, those with disabilities, and those who are extremely obese. For these special populations, goals and strategies to achieve the goals are found throughout the Strategic Plan as an integral part of obesity research. For example, the Strategic Plan includes many initiatives focused on childhood obesity, such as prevention in the pediatric primary care setting and a multi-pronged school-based prevention trial. Because of the large racial/ethnic disparities in the incidence of obesity, many of the efforts described in the Strategic Plan are directed at understanding the biologic and environmental factors contributing to such disparities and to addressing them in a culturally sensitive manner. Also relevant to health disparities is communication of the results of scientific research. The Strategic Plan addresses the importance of tailoring education and informational efforts to different populations in culturally appropriate ways that provide effective communication.

Another cross-cutting area is fostering multidisciplinary and interdisciplinary research teams. Although the first two themes (on research toward preventing and treating obesity through lifestyle approaches and medical approaches) are listed separately, they are, in fact, interdependent. Effectively addressing the obesity epidemic will require the NIH to bridge the study of the behavioral/environmental causes of obesity and the study of the genetic/biologic causes. Ultimately, the NIH seeks to create a new interdisciplinary approach in which behavioral/lifestyle interventions are informed by a deeper understanding of the biologic and genetic factors and vice versa. Successful prevention and treatment of obesity may well require combined medical and behavioral approaches in highly susceptible individuals. Given the multidimensional nature of the obesity epidemic, research efforts need to engage those with expertise in a variety of other areas, including, for example, economics, and fields relevant to the built environment and community design.

Translational research, progressing from basic science to clinical studies and from clinical trial results to community interventions, is another key cross-cutting research topic. For example, the NIH will study the effects of “social experiments” such as recent policy decisions in some schools concerning food offerings made available to the students. By obtaining data on the outcome of such policy decisions, the NIH can help policy makers develop additional actions based on data rather than on assumptions.

To continue to advance progress in many obesity research areas, efforts to improve technologies will be valuable, as will efforts addressing the availability of resources for research. The
NIH plans, for example, to encourage research to improve technologies for studying dietary intake and physical activity.

Finally, the NIH will continue activities to disseminate the results of research to the public and health care providers. It will be important that clear and appropriate communication, including messages and information about healthy eating, physical activity, and weight control strategies, reaches diverse audiences.

As noted in the Strategic Plan, the NIH will also partner with other agencies in the Department of Health and Human Services, other government departments, organizations in the private sector, and foundations and other public groups. By bringing data derived from rigorously reviewed and conducted studies to its partners, the NIH can contribute to framing the actions to address the obesity epidemic.

NIH OBESITY RESEARCH TASK FORCE WEBSITE

As one component of NIH efforts to enhance research on the major public health problem of obesity, the NIH Obesity Research Task Force has developed a new website (http://obesityresearch.nih.gov/). The primary purposes of this website are to help inform investigators about NIH funding opportunities for obesity research, to provide information on NIH-sponsored scientific meetings relevant to obesity, and to provide other information relevant to obesity research. In providing this information, the website will reflect the dynamic and ongoing planning process for obesity research at the NIH. Additionally, the Strategic Plan for NIH Obesity Research was posted on the website in draft form (after having already received substantial external input) to invite additional scientific and public comment before publication of the Strategic Plan. Finally, although the focus of the website is on research, the site also includes links to other NIH websites that provide information to the public and health professionals on weight loss, nutrition, physical activity, and health problems associated with obesity.

Through the collective efforts of the NIH Obesity Research Task Force, and the ICs they represent, the NIH will strive to bolster obesity research to improve public health.

The draft Executive Summary was prepared by Dr. Lisa J. Gansheroff, PhD (National Institute of Diabetes and Digestive and Kidney Diseases), with input from AMS and BMA, cochairs of the NIH Obesity Research Task Force. We acknowledge the members of the Task Force for their contributions to this document: National Institute of Diabetes and Digestive and Kidney Diseases, Lisa J. Gansheroff, PhD, Executive Secretary of the Task Force; Philip Smith, PhD; Susan Yanovski, MD; National Heart, Lung, and Blood Institute, Darla Danford, MPH, DSc; Karen Donato, SM, RD; Abby Ershow, ScD; Denise Simons-Morton, MD, PhD; National Cancer Institute, Rachel Ballard-Barbash, MD, MPH; National Human Genome Research Institute, Alan Guttmacher, MD; National Institute on Aging, Judith Salerno, MD, MS; National Institute on Alcohol Abuse and Alcoholism, Thomas Gentry, PhD; National Institute of Arthritis and Musculoskeletal and Skin Diseases, Gayle Lester, PhD; National Institute of Biomedical Imaging and Bioengineering, Peter Moy, PhD; National Institute of Child Health and Human Development, Gilman Grave, MD; National Institute of Dental and Craniofacial Research, Maria Canto, DDS, MPH; National Institute on Drug Abuse, Joseph Frascella, PhD; National Institute of Environmental Health Sciences, Allen Deary, PhD; National Institute of Mental Health, Richard Nakamura, PhD; National Institute of Neurological Disorders and Stroke, Merrill Miller, PhD; National Institute of Nursing Research, Yvonne Bryant, PhD, RN; National Center for Complementary and Alternative Medicine, Margaret A. Chesney, PhD; National Center on Minority Health and Health Disparities, Mireille Kanda, MD, MPH; National Center for Research Resources, David Wilde, MD, PhD; NIH Division of Nutrition Research Coordination, Van S. Hubbard, MD, PhD; Fogarty International Center, Rachel Nugent, PhD; Office of Behavioral and Social Sciences Research, Deborah Olster, PhD; Office of Dietary Supplements, Mary Frances Picciano, PhD; Office of Disease Prevention, Martina Vogel-Taylor, MT (ASCP); and Office of Research on Women’s Health, Lisa Begg, DrPH, RN. We also acknowledge the contributions of Mary Hanlon, PhD, National Institute of Diabetes and Digestive and Kidney Diseases. The authors had no conflicts of interest to report.
Genetics of common forms of obesity: a brief overview1-4

Helen N Lyon and Joel N Hirschhorn

ABSTRACT
The obesity epidemic is attributable to dietary and behavioral trends acting on a person’s genetic makeup to determine body mass and susceptibility to obesity-related disease. Common forms of obesity have a strong hereditary component, yet genetic pathways that contribute to obesity have not yet been elucidated. Many genetic association studies have been reported, but few have been successfully replicated. New research tools and large studies will lead to an understanding of genes and their interaction to cause obesity, which may help guide successful interventions and treatments. Am J Clin Nutr 2005;82(suppl):215S–7S.

KEY WORDS Obesity, genetics, association study, candidate genes, single nucleotide polymorphism, complex trait

Obesity has reached epidemic proportions in the United States and developing countries. Although the trend of decreased physical activity and increased caloric intake is probably responsible for the recent rise in obesity, it is important to understand that these trends are playing out on a background of genetic variation in the population. Each individual’s genetic background remains an important determinant of susceptibility to obesity. Discovery of the genes involved in the development of common forms of obesity, thereby identifying pathways that are causal in patients, will guide clinicians and scientists in designing more effective therapies and in identifying high-risk individuals for early intervention (1–4).

It is clear that obesity often tracks in families. Having obese relatives increases one’s risk for obesity, even if the family members do not live together or share the same patterns of exercise and food intake (5–7). Family studies and twin studies yield estimates of the fraction of the variation in the population that can be attributed to inherited variation, or the heritability (h²) (8). Estimates of heritability range from 30 to 70%, with the typical estimate at 50%, meaning about one-half of the variation in body mass within a population is a result of inherited factors (2, 5, 6). Common forms of obesity are not inherited in families in a predictable pattern like cystic fibrosis or Huntington’s disease but rather shows a complex pattern of segregation, meaning that multiple genes are involved. Because of this complex, multifactorial pattern, diseases and traits such as obesity are called complex genetic traits. A few studies have suggested that there are genes that act in a recessive manner and can explain a larger fraction of the variation in body mass. These results have not been consistently observed and may also reflect the patterns seen in early-onset, severe obesity caused by one or few genes rather than the more common polygenic, later-onset obesity observed in the general population. Thus, each of the obesity genes likely makes only a small contribution to body weight, but together inherited variation plays a large role in determining how an individual responds to the environmental factors of diet and physical activity.

What are the inherited DNA variants that affect the susceptibility to obesity? Although humans all have the same genetic material, every person’s genome is slightly different: when comparing any two copies of the same stretch of genome, about one in every 1200 bases will be different [usually a single nucleotide polymorphism (SNP)]. Most SNPs identified by comparing two chromosomes are common and shared throughout the world: 90% of such SNPs will be seen again at a frequency of at least 1% (9–11). Most of these common variants probably have no functional consequence and are essentially the equivalent of genetic dialect or random differences in spelling with no real significance. However, a few of these polymorphisms will alter the biologic function of a gene, by either affecting the structure of the protein or altering the location, amount, or time at which the protein is made. Some of these functional alterations will affect susceptibility to obesity and related diseases. A catalog of these causal variants and an appreciation of their interaction with each other and environmental factors will be crucial to designing effective interventions.

It is not yet known what types of variants (missense or regulatory, rare or common) affect complex traits such as obesity. Because most human genetic variation is common, it has been proposed based on theoretic (12, 13) and empirical (14) grounds that common variants contribute to common disease and complex traits. However, rare variants have also been proposed to play a role (15). The relative importance of missense variants has

1 From the Children’s Hospital Boston, Harvard Medical School, Broad Institute of Massachusetts Institute of Technology and Harvard University, Boston, MA.
3 Supported by National Institute of Diabetes and Digestive and Kidney Disease grant K23 DK067288-01 (HNL). JNH is a recipient of a Burroughs Wellcome Career Award in the Biomedical Sciences and a Smith Family Foundation New Investigator Award. Work leading to the unpublished data referred to in this review was supported by the Smith Family Foundation New Investigator Award and the American Diabetes Association Smith Family Foundation Pinnacle Program Project (both to JNH).
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been debated as well (16). Ideally, to have an unbiased and comprehensive search for variants with a contributory role to common obesity, we would test every variant in the genome, but such a large-scale search through even just the common variants (of which there are estimated to be 11 million) (10) is not yet practical. Thus, approaches must be taken to identify genomic regions or sets of variants that are more likely to contribute to obesity in the general population.

Two approaches have been used to date to find the variants that affect obesity, linkage analysis and association studies. Linkage analysis was used with great success in mapping genes responsible for single gene disorders. Such studies involve using multiple affected relatives to look for shared segments of DNA that are inherited more often than expected by chance, eventually narrowing the shared region to a few genes that can be tested for the presence of recognizable mutations in all of the affected relatives. Linkage studies have been applied to complex disorders such as obesity, but, in general, linkage analysis has been less successful for these multigenic diseases. Although whole genome scans often identify similar regions as being linked to obesity, the results vary greatly, probably because of the low power of linkage to find genes with modest effects or possibly differing study designs or populations. Several studies located a region on chromosome 7q31 that was found to contain the leptin gene, mutations in which cause severe obesity syndromes. Although some regions have been repeatedly implicated by linkage analysis, no genes have been found in these regions that have been seen to contribute to common obesity. As these studies are performed on larger cohorts of people, it is likely that the results will become more refined and that important obesity loci will be mapped with this method.

Association studies are another way to find genes involved in obesity. Genes and variants are selected as candidates if they have either a known or hypothesized role in metabolism, or if they are located within an area of linkage. In the simplest form of such studies, the frequency of a variant allele in a particular gene is compared in obese and non-obese individuals, or in obese individuals and their non-obese relatives. Association studies to test these functional and positional candidates have better power than linkage studies to detect the effects of common alleles of modest penetrance on complex traits such as obesity (17).

Association studies have been successfully used to identify genes for common diseases and complex traits. An example of a now well-established disease association is that of Alzheimer’s disease with the gene encoding the apolipoprotein E (ApoE). The ApoE4 variant encompasses two missense polymorphisms, and, because this genotype is found in 10–15% of the population, it is relatively common. It is found three to four times more often in people with Alzheimer’s disease, conferring a 3-fold increased risk to a carrier of this allele. Because not all people with the ApoE4 allele develop disease (and not all people with Alzheimer’s disease carry ApoE4), the allele is neither necessary nor sufficient for disease but is rather associated with higher risk. Many other associations have been reported, including for common obesity, but few have been consistently reproducible as is seen with ApoE4 and Alzheimer’s disease (3, 18).

There are several possible reasons that association studies are not replicated consistently, and it is critical to discern which is present when interpreting association studies. To explore this issue, we conducted a meta-analysis of published association studies by pooling the results from all of the follow-on studies for 25 reported associations between common genetic variants and common diseases (14). Most of these associations showed no evidence of replication in the follow-on studies. Eight of the associations showed convincing evidence of replication, and, for these, the associated variant conferred a modest effect with a less than 2-fold increase in disease risk. We concluded that most association studies are incorrect, but a fraction of reported associations are likely to be correct yet difficult to replicate with small studies that are underpowered to detect a modest effect.

More than 70 associations between body mass index or obesity and common genetic variants have been reported (3), but none have been consistently replicated. There are many interesting candidate genes in the list, including genes found to be altered in Mendelian or rare obesity syndromes, such as leptin, proopiomelanocortin, melanocortin 4 receptor (MC4R), and Bardet-Biedel syndrome loci. Bardet-Biedel syndrome can be caused by alterations in at least eight genes (3, 19, 20). We are currently investigating these eight genes for common variants, yet none have been reproducibly associated with typical obesity. There is a well-established association between rare forms of obesity and mutations in the MC4R, accounting for about 4% of early-onset severe obesity (3, 21), yet mutations in MC4R do not seem to play a prominent role in late-onset, common obesity (3, 19, 20). Rare mutations in the leptin gene cause a deficiency also leading to severe early-onset obesity. Although treatment with leptin successfully reverses this progression (22), leptin has not proven effective in treating common obesity. Identification of such rare mutations in candidate genes identifies pathways that, when disrupted, can lead to severe obesity. This suggests a general hypothesis that common variation in genes underlying severe syndromes may contribute to the common form of the disease. However, no common variants in these genes have been consistently found to be associated with common obesity in the general population.

Functional and positional candidates will continue to be investigated in the search for genes involved in the development of obesity and the related diseases. In addition, animal models will provide lists of new candidates through linkage studies, expression profiling, and transgenic strains, whereas other efforts such as expression analysis and protein interaction studies should also identify candidate genes. New molecular tools are becoming available that should expedite the testing of these genes. The complete sequence of the human genome, as well as full genome sequence in other species, are now known and are a shared resource available to all researchers. Abundant genetic variants (dbSNP) and patterns of common variation elucidated by the human HapMap will also facilitate the selection of variants to test in association studies. The HapMap should pave the way for more comprehensive, genome-wide association studies (10).

Obesity is a common disease caused by multiple factors, with heredity playing a strong causal role. There are sequence variants present in the population that increase or decrease an individual’s risk for obesity in their environment. Although we do not yet understand which pathways are altered by these variations, single gene disorders and animal models suggest a wide variety of possibilities. With new molecular tools and resources, well-powered studies can be undertaken to find common obesity genes in the future. These genes will identify root causes of obesity, potentially suggesting new therapies or interventions, and provide tools for the understanding of how people respond to their environment to become obese or remain lean.
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Teaching, learning, doing: best practices in education1–4

George L Blackburn

ABSTRACT
As many as 90 million Americans lack basic skills needed to access, understand, and use health information and services to make healthy dietary choices. Effective teaching by physicians can bridge the learning gap and arrest the epidemic of obesity. The Academy at Harvard Medical School is developing best practices in teaching that will equip future doctors to reduce health illiteracy and promote positive changes in thinking and behavior in their patients. Models of how people learn can help physicians select tasks, questions, and prompts that advance teaching and learning. To keep and use new information, adults need to integrate new ideas into existing frameworks of understanding and participate in the learning process by linking new information to what is already known. By teaching patients how to read a single food label, starting with calories, physicians can set the stage for future learning. The process of change is challenging, particularly in adults. Best practices in teaching and learning can help physicians be more effective agents of change. Am J Clin Nutr 2005;82(suppl):218S–21S.

KEY WORDS
Health literacy, agents of change, patient teaching, obesity

INTRODUCTION
The science of nutrition that underlies public health policy on the prevention and treatment of obesity has been consistently communicated to the public by those in academia, government, and industry. However, for the most part, it has failed to promote healthy eating among the public. The 74% increase in the prevalence of obesity between 1991 and 2001 could be considered a trend that reflects a breakdown in effective teaching and learning. Improvements in education are needed to close the gap between knowledge and action in the prevention and treatment of obesity.

Much of that gap can be attributed to health illiteracy, a problem almost as widespread as obesity itself. In the United States today, as many as 90 million (1) Americans lack basic skills needed to access, understand, and use health information and services to make healthy dietary choices. In recent testimony before a Senate subcommittee on the growing epidemic of childhood obesity (2), US Surgeon General Richard H. Carmona noted that many people, even educated Americans, do not know what a calorie is or how to burn it.

The data echo his claim. An estimated 129.6 million American adults, or 64%, are overweight or obese (3). Fifteen percent of children aged 6–9 y are considered overweight or obese (4). With >9 million children at increased risk of weight-related chronic diseases (5, 6), the need to translate nutrition knowledge into medical practice and personal behavior has become a necessity. Many government agencies and other organizations have responded to the crisis by urging medical schools to make communication skills a routine part of student training (1). They have also issued a call to action for US physicians to improve the way they communicate with patients.

Physicians can reduce obesity-related mortality by closing the gap between what they know and what parents and children understand about physical activity and healthy eating (7). To make healthy food choices, adults and children need easy-to-use information that fits into their busy lifestyles. By applying best practices in teaching and learning, clinicians can improve health literacy and give patients the information they need.

BEST PRACTICES IN MEDICAL EDUCATION
The Academy at Harvard Medical School (8) was recently established to advance the training of medical students by supporting the school’s most innovative and effective educators. Its focus on educational research and faculty development will give it the capacity to develop best practices in teaching, to set a standard that will help future doctors increase health literacy in their patients, and to communicate with them more effectively.

Research shows that small changes in behavior reap major dividends in personal health. Data also indicate that patients usually seek out and respect advice from their primary care physicians and that such advice can motivate them to change unhealthy behaviors (9–12). By incorporating best practices in teaching into medical school curricula, continuing medical education programs, and public health initiatives, physicians should be better able to help patients incorporate healthy changes in eating and activity levels into their daily lives.

TEACHING AND LEARNING
Models of how people learn can help physicians select tasks, questions, and other prompts that advance the teaching process.

1 From the Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA.
3 Supported by the S. Daniel Abraham Chair in Nutrition Medicine at Harvard Medical School and the Harvard Center for Healthy Living.
4 Address reprint requests and correspondence to GL Blackburn, Beth Israel Deaconess Medical Center, Feldberg 880, East Campus, 330 Brookline Avenue, Boston, MA 02215. E-mail: gblackbu@bidmc.harvard.edu.
TABLE 1
Major principles of the developmental perspective of learning

| Principle 1 | Prior knowledge is the foundation on which future knowledge is built. |
| Principle 2 | Prior knowledge must be activated for learning to take place, for students to understand and personally connect with a new concept. |
| Principle 3 | Learners must be actively involved, linking new information to what they already know. |
| Principle 4 | The links themselves are more important than the new information; the more links, and the stronger they are, the easier it becomes to apply new knowledge across a broad range of situations and choices. |
| Principle 5 | Learning cannot be context free; knowledge, and its organization into an individual’s personal construct system, is highly dependent on the context in which it was learned. |
| Principle 6 | Intrinsic motivation is associated with deep approaches to learning (eg, study required for case analysis vs multiple choice questions); it is preferable to surface approaches to learning (eg, externally motivated, rote memorization), which are associated with anxiety. |
| Principle 7 | Teaching should be geared toward making the teacher increasingly unnecessary, toward the development of the learner’s autonomy as well as intellect. |

TABLE 2
The seven levers of change

1. Reason. The rational approach uses facts, logic, and rhetoric to convince or persuade.
2. Research. Uses recent journal articles, government reports, and pertinent websites to appeal to the intellect.
3. Resonance. Restates patients’ goals and reframes the message so that it “feels right.”
4. Representational redescriptions. Repeats a point of view in many different forms (eg, linguistic, numerical, graphic) to reinforce the message.
5. Resources and rewards. Provides recognition or items of value (eg, donations to charities, money, tickets to events) for accomplishing objectives.
6. Real-world events. Leverages emotional responses to major news events to promote shifts in perspective.
7. Resistances. Identifies and defuses barriers to change, that is, resistances stemming from age, emotion, or public stance.

Responses to such cues can clarify the ideas, values, and preferences that patients bring to an office visit. In general, the more a clinician knows about a patient’s thoughts, feelings, and opinions, the more fully he or she can engage cognitive and emotional defenses and successfully bring about desired change, or at least hold open the possibility of such change.

Many schools of thought have been proposed to explain the learning process. One such example, the developmental perspective (13), defines the process as one in which an individual, faced with a need to broaden an understanding or guide future action, uses a previous interpretation of what an experience means to him or her to construct a new or revised interpretation of meaning. The developmental perspective addresses two key factors: 1) how people come to understand something, and 2) the relationship between teaching and learning. The approach, which is based on seven principles (Table 1), considers previous knowledge the most important determinant of learning. To keep, and use, new information, adults need to integrate new ideas into existing frameworks of understanding. They also need to actively participate in the learning process by linking new information to what is already known.

The links themselves are more important than the new information. The more links, and the stronger they are, the easier it becomes to apply new knowledge across a broad range of situations and problems. Integration of new information is a gradual process. The learning curve starts low and progresses upward, building on previous knowledge to extend understanding. The ability to link ideas within and between subjects and contexts, as well as between theory and practice, requires time and reflection; both are considered essential for making knowledge more accessible, transferable, and usable.

CHANGING MINDS

Strategic thinking, patience, and resourcefulness are essential for success as an agent of change. In small group or one-on-one settings, multiple factors influence a physician’s skill at changing minds. A variety of approaches (Table 2) can be used to correct misperceptions that prevent patients from realizing their personal health goals. Delivering a message many times in many different ways is one of the most powerful weapons in the armamentarium. Saying something once is not enough to prompt change, likewise with repeating one directive over and over again. It takes multiple intelligences, symbol systems, and embodiments to get an idea to “click.” Some patients, for example, will respond to cartoons and videos, some to stories and photographs, others to journal articles, government reports, and pertinent URLs.

Other ways to move patients toward healthy eating include interpersonal sensitivity, patient-important (14) encounters, confronting resistances, participating in give-and-take, and fostering bonds by engaging individuals or group members in a common enterprise. The clinician’s task is to figure out which of these, alone or in combination, is most likely to be effective in the long run for a particular individual. In general, the more a physician knows about a patient’s thoughts, feelings, and opinions, the more fully he or she can engage cognitive and emotional defenses and successfully bring about desired change.

A BLUEPRINT FOR ACTION

A treatment strategy based on the National Heart, Lung, and Blood Institute clinical guidelines on obesity (15) and the Surgeon General’s reports on physical activity (16) and obesity (17) offers clinicians an easily adapted, evidence-based blueprint that builds on previous knowledge by incorporating information about weight and exercise into discussions with patients (9). However, even with a straightforward plan, attempts to change behavior can be a slow and often frustrating process for physicians and patients (18).

Powerful forces work against change. Factors that influence the strength of resistances include time, emotion, and public stance. The longer a patient has been eating a certain way, the harder it is to change the pattern. Ideas or suggestions that spark the strongest emotional reactions in patients will bring out the strongest defenses. The more public a stance on diet and exercise,
the more difficult it is to reverse. By identifying and defusing resistances, physicians can create opportunities for change.

According to the transtheoretical or stages of change model, the process is a series of steps (Table 3), each with certain tasks and characteristics. These have been successfully incorporated into smoking cessation programs and are now being used to help people lose weight (19) and increase physical activity (20). The stages include precontemplation, contemplation, preparation, action, maintenance, and relapse/recycling. Physicians can help patients move from one level to the next by providing stage-appropriate information, support, or tools.

NO FIXED RULES

Although there are no formulas for changing minds or behaviors, certain similarities apply to most individuals. Data indicate that people learn and change through successive approximation (21), and that they need to hear a particular message several times, and in a variety of ways, before it can become an impetus to action. Physicians can begin delivering effective messages by teaching patients how to read a single food label (Figure 1), starting with calories. It takes little time or effort to explain what a calorie is and how it affects body weight.

That basic lesson, which can establish the previous knowledge that makes future learning possible, will be reinforced by the Food and Drug Administration’s recently launched Calories Count campaign (http://www.fda.gov/oc/initiatives/obesity) (22). The simple message that “calories in must equal calories out” is the foundation for a national education effort to encourage Americans to take small steps to fight obesity (http://www.small-step.gov/). As part of that initiative, the Food and Drug Administration will also evaluate how the Nutrition Facts panel on food labels can be revised to highlight the critical role calories play in consumers’ diets (Figure 2).

THE CHALLENGES OF CHANGE

The job of promoting change is rarely a matter of straightforward persuasion; more likely, it is an ongoing effort to create conditions for change and keep hope alive (23). Studies show that the process of change is gradual, particularly in adults. Success requires years of learning (18). Most people are unable to convert new behavior into habitual practice without guided application over a significant length of time. It takes an estimated 10 y to become an expert in any given discipline. In the Look AHEAD (Action For Health in Diabetes) trial, for example, obese patients are randomly assigned to Lifestyle or Diabetes Support and Education interventions for 4 y, with 7.5 y of follow-up (24). Similarly, the Women’s Intervention Nutrition Study protocol includes randomization to intensive dietary intervention with long-term counseling, followed by monthly group sessions (25).

Challenges associated with modifying patients’ behavior are enormous and extend far beyond persuading them to take their pills (26). Best practices in teaching should help physicians meet these challenges with new skills that enable them to improve communication and be more effective agents of change. Public health campaigns to increase health literacy are also expected to help by empowering people to use nutrition aids and information [eg, the Dietary Guidelines (27), the Food Guide Pyramid (28), and the Healthy Eating Index (29)] to make healthy food choices. To date, these approaches lack scientific evidence to support their use. Randomized trials are needed to find out whether medical students who learn communication skills do a better job at increasing health literacy than those who do not. The same holds true with learning theory and the psychology of change; data have yet to establish whether exposure to such material will make clinicians more effective agents of change. Similarly, prospective trials are needed to measure the effectiveness of public health campaigns to improve the food choices and weight maintenance in American adults and children.

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**TABLE 3**
The stages of change model

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
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<tbody>
<tr>
<td>Precontemplation</td>
<td>Not ready for change</td>
</tr>
<tr>
<td>Contemplation</td>
<td>Thinking about change</td>
</tr>
<tr>
<td>Preparation</td>
<td>Getting ready to make a change, planning, and commitment</td>
</tr>
<tr>
<td>Action</td>
<td>Making the change, implementing the plan, taking the action</td>
</tr>
<tr>
<td>Maintenance</td>
<td>Sustaining and integrating behavior change</td>
</tr>
<tr>
<td>Relapse/recycling</td>
<td>Slipping back to previous behavior and reentering the cycle of change</td>
</tr>
</tbody>
</table>

**FIGURE 1.** Emphasizing calories (22). Calories Count is the key message in the Food and Drug Administration’s campaign to fight obesity in America.

**FIGURE 2.** Labeling calories: the entire container (22). Counting calories is critical for people who are trying to achieve and maintain a healthy weight.

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**Nutrition Facts**

**Table 3**

<table>
<thead>
<tr>
<th>Serving Size</th>
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<th>Trans Fat</th>
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<th>Sodium</th>
<th>Total Carbohydrate</th>
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<th>Sugars</th>
<th>Fiber</th>
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<td>5%</td>
</tr>
<tr>
<td>200 mL</td>
<td>110</td>
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**Nutrition Facts**

**Table 4**

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REFERENCES


Long-term weight loss maintenance\textsuperscript{1–4}

Rena R Wing and Suzanne Phelan

ABSTRACT
There is a general perception that almost no one succeeds in long-term maintenance of weight loss. However, research has shown that \(\approx20\%\) of overweight individuals are successful at long-term weight loss when defined as losing at least 10\% of initial body weight and maintaining the loss for at least 1 y. The National Weight Control Registry provides information about the strategies used by successful weight loss maintainers to achieve and maintain long-term weight loss. National Weight Control Registry members have lost an average of 33 kg and maintained the loss for more than 5 y. To maintain their weight loss, members report engaging in high levels of physical activity (\(\approx1\) h/d), eating a low-calorie, low-fat diet, eating breakfast regularly, self-monitoring weight, and maintaining a consistent eating pattern across weekdays and weekends. Moreover, weight loss maintenance may get easier over time; after individuals have successfully maintained their weight loss for 2–5 y, the chance of longer-term success greatly increases. Continued adherence to diet and exercise strategies, low levels of depression and disinhibition, and medical triggers for weight loss are also associated with long-term success. National Weight Control Registry members provide evidence that long-term weight loss maintenance is possible and help identify the specific approaches associated with long-term success. \textit{Am J Clin Nutr} 2005;82(suppl):222S–5S.

KEY WORDS Weight maintenance, successful weight loss, weight regain, obesity, National Weight Control Registry

SUCCESSFUL WEIGHT LOSS MAINTENANCE
The perception of the general public is that no one ever succeeds at long-term weight loss. This belief stems from Stunkard and McLaren-Hume’s 1959 study of 100 obese individuals, which indicated that, 2 y after treatment, only 2\% maintained a weight loss of 9.1 kg (20 lb) or more (1). More recently, a New England Journal of Medicine editorial titled \textit{Losing Weight: An Ill-Fated New Year’s Resolution} (2) echoed the same pessimistic message.

The purpose of this paper is to review the data on the prevalence of successful weight loss maintenance and then present some of the major findings from the National Weight Control Registry (NWCR), a database of more than 4000 individuals who have indeed been successful at long-term weight loss maintenance.

DEFINING “SUCCESSFUL WEIGHT LOSS MAINTENANCE”
Wing and Hill (3) proposed that successful weight loss maintainers be defined as “individuals who have intentionally lost at least 10\% of their body weight and kept it off at least one year.” Several aspects of this definition should be noted. First, the definition requires that the weight loss be intentional. Several recent studies indicate that unintentional weight loss occurs quite frequently and may have different causes and consequences than intentional weight loss (4, 5). Thus, it is important to include intentionality in the definition. The 10\% criterion was suggested because weight losses of this magnitude can produce substantial improvements in risk factors for diabetes and heart disease. Although a 10\% weight loss may not return an obese to a non-obese state, the health impact of a 10\% weight loss is well documented (6). Finally, the 1-y duration criterion was proposed in keeping with the Institute of Medicine criteria (7). Clearly, the most successful individuals have maintained their weight loss longer than 1 y, but selecting this criterion may stimulate research on the factors that enable individuals who have maintained their weight loss for 1 y to maintain it through longer intervals.

PREVALENCE OF SUCCESSFUL WEIGHT LOSS MAINTENANCE
There are very few studies that have used this definition to estimate the prevalence of successful weight loss maintenance. McGuire et al (8) reported results of a random digit dialing survey of 500 adults, 228 of whom were overweight or obese [body mass index (BMI) \(\geq27\) kg/m\(^2\)] at their maximum nonpregnant weight. Of these 228, 47 (20.6\%) met the criteria for successful weight loss maintenance: they had intentionally lost at least 10\% of their body weight and maintained it for at least 1 y. On average, these 47 individuals had lost 20.7 ± 14.4 kg (45.5 lb; 19.5 ± 10.6\% from maximum weight) and kept it off for 7.2 ± 8.5 y; 28 of the 47 had reduced to normal weight (BMI <27 kg/m\(^2\)).

Survey data such as these have the perspective of a person’s entire lifetime and thus may include many weight loss attempts, some of which were successful and some unsuccessful. It is more typical to assess “success” during one specific weight loss bout. In standard behavioral weight loss programs, participants lose an average of 7–10\% (7–10 kg) of their body weight at the end of the

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\textsuperscript{3} Supported by Community Foundation for Southeastern Michigan and National Institutes of Health grant RO1 DK066787-01.
\textsuperscript{4} Address reprint requests and correspondence to RR Wing, Weight Control and Diabetes Research Center, 196 Richmond Street, Providence, RI 02903. E-mail: rwing@lifespan.org.
initial 6-mo treatment program and then maintain a weight loss of \( \approx 5-6 \) kg (5–6%) at 1-y follow-up. Only a few studies have followed participants for longer intervals; in these studies, \( \approx 13-20\% \) maintain a weight loss of 5 kg or more at 5 y. In the Diabetes Prevention Program (9), \( \approx 1000 \) overweight individuals with impaired glucose tolerance were randomly assigned to an intensive lifestyle intervention. The average weight loss of these participants was 7 kg (7%) at 6 mo; after 1 y, participants maintained a weight loss of \( \approx 6 \) kg (6%), and, at 3 y, they maintained a weight loss of \( \approx 4 \) kg (4%). At the end of the study (follow-up ranging from 1.8 to 4.6 y; mean, 2.8 y), 37\% maintained a weight loss of 7\% or more.

Thus, although the data are limited and the definitions varied across studies, it appears that \( \approx 20\% \) of overweight individuals are successful weight losers.

**THE NATIONAL WEIGHT CONTROL REGISTRY**

Although it is often stated that no one ever succeeds in weight loss, we all know some people who have achieved this feat. In an effort to learn more about those individuals who have been successful at long-term weight loss, Wing and Hill (10) established the National Weight Control Registry in 1994. This registry is a self-selected population of more than 4000 individuals who are age 18 or older and have lost at least 13.6 kg (30 lb) and kept it off at least 1 y. Registry members are recruited primarily through newspaper and magazine articles. When individuals enroll in the registry, they are asked to complete a battery of questionnaires detailing how they originally lost the weight and how they now maintain this weight loss. They are subsequently followed annually to determine changes in their weight and their weight-related behaviors.

The demographic characteristics of registry members are as follows: 77\% are women, 82\% are college educated, 95\% are Caucasian, and 64\% are married. The average age at entry to the registry is 46.8 y. About one-half of registry members report having been overweight as a child, and almost 75\% have one or two parents who are obese.

Participants self-report their current weight and their maximum weight. Previous studies suggest that such self-reported weights are fairly accurate (slightly underestimating actual weight) (11, 12). In the NWCR, participants are asked to identify a physician or weight loss counselor who can provide verification of the weight data. When, in a subgroup of participants, the information provided by participants was compared with that given by the professional, the self-report information was found to be very accurate.

Participants in the registry report having lost an average of 33 kg and have maintained the minimum weight loss (13.6 kg) for an average of 5.7 y. Thirteen percent have maintained this minimum weight loss for more than 10 y. The participants have reduced their BMI from 36.7 kg/m\(^2\) at their maximum to 25.1 kg/m\(^2\) currently. Thus, by any criterion, these individuals are clearly extremely successful.

Previously, we reported information about the way in which registry participants lost their weight (10); interestingly, about one-half (55.4\%) reported receiving some type of help with weight loss (commercial program, physician, nutritionist), whereas the others (44.6\%) reported losing the weight entirely on their own. Eighty-nine percent reported using both diet and physical activity for weight loss; only 10\% reported using diet only, and 1\% reported using exercise only for their weight loss. The most common dietary strategies for weight loss were to restrict certain foods (87.6\%), limit quantities (44\%), and count calories (43\%). Approximately 25\% counted fat grams, 20\% used liquid formula, and 22\% used an exchange system diet. Thus, there is variability in how the weight loss was achieved (except that it is almost always by diet plus physical activity).

The earliest publication regarding the registry documented the behaviors that the members \((n = 784)\) were using to maintain their weight loss (10). Three strategies were reported very consistently: consuming a low-calorie, low-fat diet, doing high levels of physical activity, and weighing themselves frequently. Recently, a fourth behavior was identified: consuming breakfast daily (13). Each of these behaviors is described below. Registry members reported eating 1381 kcal/d, with 24\% of calories from fat. In interpreting their data, it is important to recognize that 55\% of registry members report that they are still trying to lose weight and to consider that dietary intake is typically underestimated by 20–30\%. Thus, registry members are probably eating closer to 1800 kcal/d. However, even with this adjustment, it is apparent that registry members maintain their weight loss by continuing to eat a low-calorie, low-fat diet.

More recently, we have examined other aspects of their diet. Of particular interest is the fact that 78\% of registry members report eating breakfast every day of the week (13). Only 4\% report never eating breakfast. The typical breakfast is cereal and fruit. Registry members also report consuming 2.5 meals/wk in restaurants and 0.74 meals/wk in fast food establishments.

Another characteristic of NWCR members is high levels of physical activity. Women in the registry reported expending an average of 2545 kcal/wk in physical activity, and men report an average of 3293 kcal/wk (10). These levels of activity would represent \( \approx 1 \) h/d of moderate-intensity activity, such as brisk walking. The most common activity is walking, reported by 76\% of the participants. Approximately 20\% report weight lifting, 20\% report cycling, and 18\% report aerobics.

Registry members also reported frequent monitoring of their weight (10). More than 44\% report weighing themselves at least once a day, and 31\% report weighing themselves at least once a week. This frequent monitoring of weight would allow these individuals to catch small weight gains and hopefully initiate corrective behavior changes.

The vigilance regarding body weight can be seen as one aspect of the more general construct of cognitive restraint (ie, the degree of conscious control exerted over eating behaviors). Registry members are asked to complete the Three Factor Eating Inventory (14), which includes a measure of cognitive restraint. Registry members scored high on this measure (mean of 7.1), with levels similar to those seen in patients who have recently completed a treatment program for obesity, although not as high as eating-disordered patients. These findings suggest that successful weight loss maintainers continue to act like recently successful weight losers for many years after their weight loss.

**FACTORS ASSOCIATED WITH WEIGHT REGAIN**

Registry participants are followed over time to identify variables related to continued success at weight loss and maintenance. Findings from the initial follow-up study (15) indicated that, after 1 y, 35\% gained 2.3 kg (5 lbs) or more (7 kg on
Participants who regained weight (>2.3 kg) were compared with those who continued to maintain their body weight to examine whether there were any baseline characteristics that could distinguish the two groups. The single best predictor of risk of regain was how long participants had successfully maintained their weight loss (Table 1). Individuals who had kept their weight off for 2 y or more had markedly increased odds of continuing to maintain their weight over the following year. This finding is encouraging because it suggests that, if individuals can succeed at maintaining their weight loss for 2 y, they can reduce their risk of subsequent regain by nearly 50%.

Another predictor of successful weight loss maintenance was a lower level of dietary disinhibition, which is a measure of periodic loss of control of eating. Participants who had fewer problems with disinhibition (ie, scores <6 on the Eating Inventory subscale [14]) were 60% more likely to maintain their weight over 1 y. Similar findings were found for depression, with lower levels of depression related to greater odds of success. These findings point to the importance of both emotional regulation skills and control over eating in long-term successful weight loss.

Several key behavior changes that occurred over the year of follow-up also distinguished maintainers from regainers. Not surprisingly, those who regained weight reported significant decreases in their physical activity, increases in their percentage of calories from fat, and decreases in their dietary restraint. Thus, a large part of weight regain may be attributable to an inability to maintain healthy eating and exercise behaviors over time. The findings also underscore the importance of maintaining behavior changes in the long-term maintenance of weight loss.

### Triggering events

Another variable that has been examined in the registry is the presence of a “triggering event” leading to participants’ successful weight loss. Most registry participants reported a trigger for their weight loss (83%). Medical triggers were the most common (23%), followed by reaching an all time high in weight (21.3%), and seeing a picture or reflection of themselves in the mirror (23%), followed by reaching an all time high in weight (21.3%), and seeing a picture or reflection of themselves in the mirror (23%), followed by reaching an all time high in weight (21.3%), and seeing a picture or reflection of themselves in the mirror (23%), followed by reaching an all time high in weight (21.3%), and seeing a picture or reflection of themselves in the mirror (23%), followed by reaching an all time high in weight (21.3%), and seeing a picture or reflection of themselves in the mirror (23%), followed by reaching an all time high in weight (21.3%), and seeing a picture or reflection of themselves in the mirror (23%), followed by reaching an all time high in weight (21.3%), and seeing a picture or reflection of themselves in the mirror (23%), followed by reaching an all time high in weight (21.3%), and seeing a picture or reflection of themselves in the mirror (23%), followed by reaching an all time high in weight (21.3%), and seeing a picture or reflection of themselves in the mirror (23%), followed by reaching an all time high in weight (21.3%), and seeing a picture or reflection of themselves in the mirror (23%), followed by reaching an all time high in weight (21.3%), and seeing a picture or reflection of themselves in the mirror (23%), followed by reaching an all time high in weight (21.3%), and seeing a picture or reflection of themselves in the mirror (23%), followed by reaching an all time high in weight (21.3%), and seeing a picture or reflection of themselves in the mirror (23%).

Because medical triggers have been shown to promote long-term behavior change in other areas of behavioral medicine (16), we examined whether individuals who reported medical triggers were more successful than those who reported nonmedical triggers or no triggers. A medical trigger was defined broadly and included, for example, a doctor telling the participant to lose weight and/or a family member having a heart attack. Findings indicated that people who had medical reasons for weight loss also had better initial weight losses and maintenance (17). Specifically, those who said they had a medical trigger lost 36 kg, whereas those who had no trigger (17.1%) or a nonmedical trigger (59.9%) lost 32 kg. Medical triggers were also associated with less regain over 2 y of follow-up. Those with medical triggers gained 4 kg (≈2 kg/y), whereas those with other or no medical triggers gained at a significantly faster rate, averaging 6 kg in both groups.

These findings are intriguing because they suggest that the period following a medical trigger may be an opportune time to initiate weight loss to optimize both initial and long-term weight loss outcomes.

### Dieting consistency

The topic of dieting consistency was also recently examined in the registry. Participants were asked whether they maintained the same diet regimen across the week and year, or if they tended to diet more strictly on weekdays and/or nonholidays (18). Few people said they dieted more strictly on the weekend compared with the rest of the week (2%) or during holidays compared with the rest of the year (3%). Most participants reported that their eating was the same on weekends and weekdays (59%) and on holidays/vacations and the rest of the year (45%). The remaining groups reported that they were stricter during the week than on weekends (39%) and during nonholiday times compared with holidays (52%).

We evaluated whether maintaining a consistent diet was related to subsequent weight regain after 2 y. Interestingly, results indicated that participants who reported a consistent diet across the week were 1.5 times more likely to maintain their weight within 5 lb over the subsequent year than participants who dieted more strictly on weekdays. A similar relationship emerged between dieting consistency across the year and subsequent weight regain; individuals who allowed themselves more flexibility on holidays had greater risk of weight regain. Allowing for flexibility in the diet may increase exposure to high-risk situations, creating more opportunity for loss of control. In contrast, individuals who maintain a consistent diet regimen across the week and year appear more likely to maintain their weight loss over time.

### Recovery from relapse

We also examined different patterns of weight change among registry participants followed over time. We were particularly interested in evaluating whether participants who gained weight between baseline and year 1 were able to recover over the subsequent year. We found that few people (11%) recovered from even minor lapses of 1–2 kg. Similarly, magnitude of weight regain at year 1 was the strongest predictor of outcome from year 0 to 2. Participants who gained the most weight at year 1 were the least likely to re-lose weight the following year, both when “recovery” was defined as a return to baseline weight or as re-losing at least 50% of the year 1 gain.

Although participants gained weight and recovery was uncommon, the regains were modest (average of 4 kg at 2 y), and the vast majority of participants (96%) remained >10% below their maximum lifetime weight, which is considered “successful” by current obesity treatment standards.

These findings, nonetheless, suggest that reversing weight regain appears most likely among individuals who have gained...
the least amount of weight. Preventing small regains from turning into larger relapses appears critical to recovery among successful weight losers.

SUMMARY

Results of random digit dial surveys indicate that approximately 20% of people in the general population are successful at long-term weight loss maintenance. These data, along with findings from the National Weight Control Registry, underscore the fact that it is possible to achieve and maintain significant amounts of weight loss.

Findings from the registry suggest six key strategies for long-term success at weight loss: 1) engaging in high levels of physical activity; 2) eating a diet that is low in calories and fat; 3) eating breakfast; 4) self-monitoring weight on a regular basis; 5) maintaining a consistent eating pattern; and 6) catching “slips” before they turn into larger regains. Initiating weight loss after a medical event may also help facilitate long-term weight control.

Additional studies are needed to determine the factors responsible for registry participants’ apparent ability to adhere to these strategies for a long period of time in the context of a “toxic” environment that strongly encourages overeating and sedentary lifestyles.

RRW is the cofounder of the National Weight Control Registry (with James O Hill). RRW coauthored the manuscript with SP, who is a coinvestigator of the National Weight Control Registry. RRW and SP have no financial or personal interest in the organizations sponsoring this research.

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Physical activity considerations for the treatment and prevention of obesity1–4

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ABSTRACT

Obesity, one of the most pressing public health concerns, is the result of a positive energy balance. Overweight and obesity present significant public health concerns because of the link with numerous chronic health conditions. Excess body weight is a result of an imbalance between energy intake and energy expenditure. Physical activity is the most variable component of energy expenditure and therefore has been the target of behavioral interventions to modify body weight. It appears that physical activity is an important component on long-term weight control, and therefore adequate levels of activity should be prescribed to combat the obesity epidemic. Although there is evidence that 30 min of moderate-intensity physical activity may improve health outcomes, the amount of physical activity that may be necessary to control body weight may be >30 min/d. There is a growing body of scientific literature suggesting that at least 60 min of moderate-intensity physical activity may be necessary to maximize weight loss and prevent significant weight regain. Moreover, adequate levels of physical activity appear to be important for the prevention of weight gain and the development of obesity. Physical activity also appears to have an independent effect on health-related outcomes when compared with body weight, suggesting that adequate levels of activity may counteract the negative influence of body weight on health outcomes. Thus, it is important to target intervention strategies to facilitate the adoption and maintenance of an adequate amount of physical activity to control body weight. Am J Clin Nutr 2005;82(suppl):226S–9S.

KEY WORDS Exercise, physical activity, energy expenditure, obesity, weight control

INTRODUCTION

It is estimated that in excess of 65% of adults are classified as overweight (body mass index ≥25.0 kg/m²), with ≈30% of adults classified as obese (body mass index ≥30 kg/m²) (1). These prevalence rates of overweight and obesity reflect a significant increase in these prevalence rates over the past few decades. These increasing rates of overweight and obesity are of concern because of the demonstrated association with numerous chronic conditions, which include cardiovascular disease, diabetes, and various forms of cancer (2). Therefore, it is critical to develop and implement effective interventions for the prevention and treatment of excess body weight.

WHAT IS THE ROLE OF PHYSICAL ACTIVITY IN WEIGHT LOSS?

The key to managing body weight is energy balance. When energy expenditure is equal to energy intake, theoretically body weight will be maintained, which should be the goal for prevention of initial weight gain or prevention of weight regain after weight loss. However, to promote weight loss, it is necessary to create an energy imbalance that elicits an energy deficit. Physical activity in the form of structured exercise contributes to the creation of an energy deficit by increasing total energy expenditure, and this can promote weight loss. However, although exercise is an important component of weight loss interventions, it is important to understand the magnitude of the contribution of exercise to weight loss within clinical interventions.

Short-term interventions, which are typically 6 mo or less in duration, have examined the effect of exercise alone and in combination with reductions in energy intake on changes in body weight. When compared, these studies have demonstrated that reductions in energy intake (eg, diet) have a greater impact on body weight than changes in energy expenditure via exercise, with the combination of diet plus exercise having the greatest impact on weight loss. For example, Hagan et al (3) reported reductions in body weight of 11.4, 8.4, and 0.3% in males participating in 12 wk of diet plus exercise, diet alone, or exercise alone, respectively. A similar pattern of weight losses of 7.5, 5.5, and 0.6% was observed in women engaging in the same interventions. The more recent review of the literature conducted as part of the clinical guidelines developed by the National Heart, Lung, and Blood Institute has consistently identified a similar pattern of results in numerous short-term studies of diet plus exercise, diet alone, and exercise alone (2). Despite the results of clinical intervention studies that demonstrate that changes in energy intake may have a greater impact on body weight than exercise alone, there are studies that suggest that exercise can be as effective as diet for precipitating initial weight loss. For example, Ross et al (4) compared the effect of a 700 kcal/d energy deficit on 3 mo weight loss when produced via reduction in energy intake versus an increase in energy expenditure (exercise). The investigators reported comparable weight losses of 7.6 kg in the energy restriction and energy expenditure

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groups, respectively. These results provide some evidence that exercise can be as effective as changes in diet for producing weight loss. However, it should be noted that a 90.7 kg (200 lb) individual would need to engage in ≈115.7 min/d (1 h 57 min/d) of brisk walking (four metabolic equivalents) to expend 700 kcal/d in exercise to produce this magnitude of weight loss. These data highlight why clinical interventions typically report greater initial weight loss with changes in diet versus changes in exercise, because most overweight and obese individuals may be unlikely to engage in sufficient levels of exercise to produce the magnitude of weight loss typically observed with reductions in energy intake.

The benefits of exercise for weight control may be best observed when exercise continues as part of the treatment plan beyond the initial weight loss period, which is typically 6 mo in duration. Studies have demonstrated that exercise alone can have a significant impact on body weight when maintained for ≥12 mo (5, 6). Moreover, it has been shown that exercise may contribute to additional weight loss beyond the weight reduction observed within the initial 6 mo of treatment (7). Of greater importance is the observation that individuals who are capable of maintaining their weight loss long term also report engaging in exercise as part of their behavioral treatment program (7–9). An interesting observation in a study conducted by McGuire et al (10) was that individuals in the National Weight Control Registry who reduced their level of leisure-time physical activity also reported weight regain across a 1-y period. Thus, for exercise to be effective long term, it will be important to implement strategies that will facilitate the maintenance of the exercise behavior long term in overweight and previously overweight individuals.

Another important consideration is that exercise does not operate in isolation to enhance long-term maintenance of weight loss. Rather, exercise functions in concert with other important behaviors to contribute to the successful maintenance of weight loss. For example, Jakicic et al (11) reported that exercise is one behavior in a constellation of behaviors that contributes to long-term maintenance of weight loss. Across an 18-mo intervention, it was reported that individuals who maintained a greater magnitude of weight loss were engaging in both adequate amounts of exercise and healthy eating behaviors associated with weight control. Thus, the maintenance of weight loss may be a result of a constellation of behaviors, of which exercise is one of the important behaviors.

**WHAT IS THE ROLE OF PHYSICAL ACTIVITY IN THE PREVENTION OF WEIGHT GAIN?**

Because of the impact on energy balance, physical activity may contribute to the prevention of weight gain, which may slow the increasing prevalence rates of obesity in the United States. Data from observation studies support the potential importance of exercise in the prevention of weight gain. DiPietro et al (12) reported that a modest increase in fitness, which most likely reflects regular participation in exercise, prevented weight gain across a 4-y period in adults. Similarly, Sherwood et al (13) reported that exercise may have contributed to the prevention of weight gain in the Pound of Prevention Study. There are ongoing clinical trials to further examine the dose of physical activity to prevent weight gain. However, the currently available data provides initial support for the inclusion of exercise in efforts to curtail the increase in body weight that has been observed over the past few decades and has contributed to the obesity epidemic.

Of interest is the effect of interventions on the prevention of weight gain. Data from the recently completed STRIDDE study reported that control subjects gained weight, whereas those who participated in exercise had modest decreases in body weight and measures of body fatness, and this appears to occur in a dose-response manner (14). Regardless of these results, the optimal magnitude of exercise that is most effective for prevention of weight gain is still being debated. Therefore, it may be advantageous to initially target 150 min/wk of exercise, because this is the minimal recommended level of physical activity to promote improvements in health and to increase levels of exercise as needed to promote the prevention of weight gain.

**ARE THERE HEALTH BENEFITS FOR OVERWEIGHT ADULTS INDEPENDENT OF BODY WEIGHT?**

There is a growing body of knowledge to support the independent effects of physical activity and improvements in cardiorespiratory fitness on health-related outcomes (15, 16). It is important to acknowledge that overweight and obese adults may realize significant improvements in health-related outcomes independent of weight loss (17–19). Wei et al (19) reported that cardiorespiratory fitness was a significant predictor of cardiovascular disease and all-cause mortality across categories of normal weight, overweight, and obese men. Similar findings have been reported for women, with low levels of cardiorespiratory fitness being a stronger predictor than body mass index of all-cause mortality (19). This pattern of results has been confirmed in a recent study of women in the Women’s Ischemic Syndrome Evaluation study, which reported lower coronary artery disease risk factors, angiograph coronary artery disease, and cardiovascular events with increasing levels of cardiorespiratory fitness, with no independent effect of body weight on these outcomes (20). These findings support the importance of improving physical activity and cardiorespiratory fitness in overweight and obese adults, and this should be one of the focal points of interventions for individuals in this population.

**WHAT IS THE DOSE OF PHYSICAL ACTIVITY TO MAXIMIZE WEIGHT CONTROL?**

To realize the benefits of physical activity for weight control, individuals need to participate in adequate levels of physical activity. It is commonly accepted that the equivalent of at least 150 min/wk of moderate-intensity physical activity is necessary to realize improvements in health-related outcomes (16, 21). However, higher levels of physical activity may be necessary to improve long-term weight loss outcomes. For example, in two separate studies, Jakicic et al (7, 22) have reported that long-term weight loss was improved in overweight and obese women with the addition of 200–300 min/wk of physical activity. These findings are similar to the results reported by Schoeller et al (23) who demonstrated that weight loss maintenance was improved when individuals engaged in the equivalent of ≈65 min/d of moderate-intensity physical activity. It has also been demonstrated that ≥2000 kcal/wk of physical activity may improve long-term weight loss outcomes (9, 24). Thus, although there may be health-related benefits associated with at least 150 min/wk of moderate-intensity physical activity (16, 21), there is a growing
body of knowledge to support higher levels of physical activity for improving long-term weight loss outcomes. Blair et al (25) have concluded recently that, although 30 min/d of moderate-intensity physical activity may provide substantial health benefits, “this dose of exercise may be insufficient to prevent unhealthful weight gain for some persons who need additional exercise or caloric restriction to minimize the likelihood of additional weight gain.” Thus, encouraging levels of physical activity greater than the minimum public health recommendation (150 min/wk) may be necessary for improving long-term weight loss outcomes, and this is supported by the physical activity recommendations of the Institute of Medicine (26), American College of Sports Medicine (27), and the International Association for the Study of Obesity (28).

ADDITIONAL PHYSICAL ACTIVITY CONSIDERATIONS

Resistance exercise

Research has continued to focus on the potential benefits of resistance exercise for overweight and obese individuals. Unfortunately, it does not appear that resistance exercise has clear advantages over other forms of exercise for enhancing weight loss outcomes, and this is supported in a recent review of the literature conducted by Donnelly et al (29). Interventions that have combined dietary energy restriction with resistance exercise have shown no clear weight loss advantage when compared with energy restriction combined with other forms of exercise. A potential limitation of these studies is the relatively short-term intervention periods, with most not exceeding 6 mo in duration. However, in one of the few long-term studies to examine the effect of resistance exercise on weight loss, Wadden et al (30) reported no improvement in weight loss across a 40-wk intervention period with the inclusion of resistance exercise. Donnelly et al (29) also concluded that resistance exercise in the absence of restriction in energy intake has minimal impact of changes in absolute body weight, which may be partially explained by modest increases in fat-free mass combined with modest reductions in fat mass.

The advantage of including resistance exercise in interventions for the prevention and treatment of overweight and obesity may be the known benefits of resistance exercise on muscular strength. This may prove to be advantageous for improving function and the ability to perform activities of daily living in overweight and obese individuals. For example, the ability to lift one’s own body weight may facilitate opportunities for physical activity in these individuals, which may improve physical activity levels and/or enhance function, which results in improvement in quality of life. Thus, the addition of resistance exercise may prove beneficial for overweight and obese adults for reasons other than the impact of this form of exercise on weight loss.

Intermittent exercise

Perceived lack of time is a commonly reported barrier to participation in physical activity. However, there is some evidence that recommending the accumulation of physical activity across multiple intermittent periods throughout the day should be considered as an alternative intervention approach to facilitate the adoption of physical activity in previously sedentary overweight adults (22, 31). Jakicic et al (22, 31) reported that the strategy to include intermittent periods of physical activity throughout the day was effective for improving physical activity participation in overweight women across a 20-wk behavioral weight loss program, and this may have contributed to a trend for improved weight loss when compared with continuous periods of physical activity (ie, 40 continuous minutes). However, despite initial improvements in physical activity, to date this strategy has not been shown to improve long-term physical activity participation in overweight adults when compared with continuous exercise (22, 32). Therefore, this strategy may be most effective for improving the initial adoption of physical activity in overweight and obese individuals. This should be considered as an alternative strategy that may facilitate participation in physical activity during periods when there are barriers to participation in more traditional periods of physical activity (ie, 30–60 continuous minutes).

Lifestyle approaches to increasing physical activity

An additional alternative to tradition forms of exercise may be lifestyle approaches to physical activity. This approach emphasizes seeking opportunities in one’s lifestyle to engage in physical activity. An example of a lifestyle physical activity is to use walking rather than motorized alternatives (ie, automobiles, elevators, and escalators) for the purpose of transportation and mobility. It has been reported that lifestyle approaches to physical activity may result in cardiorespiratory fitness and body weight changes that are similar to what is observed with more traditional forms of exercise (33, 34). However, despite investigators describing the intervention that was prescribed to participants in these studies, the actual mode, duration, and intensity of the lifestyle activity in which the participants engaged has not been reported. Therefore, it is difficult to determine whether a lifestyle approach to physical activity resulted in varying patterns of physical activity that is significantly different from what is commonly observed with more traditional approaches to exercise. Thus, additional research may be necessary to understand the true impact of specific forms and patterns of lifestyle physical activity on body weight, cardiorespiratory fitness, and additional health-related outcomes in overweight adults.

There is an increasing use of pedometers to promote the increase in steps walked, and promoting lifestyle forms of physical activity may enhance the accumulation of steps. Currently, it is suggested that the accumulation of at least 10 000 steps per day may be associated with improvements in health-related parameters (35). Assuming that the average adult accumulates 2000 steps for walking 1 mile, the average adult would need to add at least 2 miles of walking per day to their current level of physical activity to achieve the goal of 10 000 steps per day (35). This is consistent with current public health recommendations to increase physical activity by at least 30 min/d (16, 21). However, it has been suggested that progressing to a higher step goal, or concurrent reductions in energy intake, may be necessary to reduce body weight in overweight and obese adults (36).

SUMMARY

Obesity is a significant public health problem that requires the development and implementation of effective interventions for both prevention and treatment. Physical activity appears to be an
important behavior that may prevent weight gain and significantly contribute to enhancing long-term weight loss and reducing health-risks associated with numerous chronic health conditions. There is strong scientific support for physical activity to be combined with modifications to energy intake as the most effective behavioral approach for addressing the obesity epidemic. Moreover, although 30 min/d of moderate-intensity physical activity may result in significant improvements in health, it appears that progressing to at least 60 min of physical activity may be necessary for enhancing long-term weight loss outcomes. Thus, it is important to target interventions to improve physical activity participation in overweight and obese individuals to maximize weight control and corresponding improvements in health-related outcomes.

JMJ and ADO both contributed to the development of this manuscript. JMJ is on the scientific advisory boards for the Coca-Cola Beverage Institute for Health and Wellness, the Calorie Control Council (caloriescount.com), BodyMedia Inc, and One Rock Technologies Inc. ADO is on the scientific advisory board for One Rock Technologies Inc.

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Behavioral treatment of obesity

Gary D Foster, Angela P Makris, and Brooke A Bailer

ABSTRACT

Behavioral treatment is an approach used to help individuals develop a set of skills to achieve a healthier weight. It is more than helping people to decide what to change; it is helping them identify how to change. The behavior change process is facilitated through the use of self-monitoring, goal setting, and problem solving. Studies suggest that behavioral treatment produces weight loss of 8–10% during the first 6 mo of treatment. Structured approaches such as meal replacement and food provision have been shown to increase the magnitude of weight loss. Most research on behavioral treatment has been conducted in university-based clinic programs. Although such studies are important, they tell us little about the effectiveness of these approaches in settings outside of specialized clinics. Future research might focus more on determining how these behavioral techniques can be best applied in a real-world setting.

INTRODUCTION

Historically, behavioral treatment of obesity developed from the belief that obesity was the result of maladaptive eating and exercise habits, which could be corrected by the application of learning principles (1). Today, investigators realize that body weight is affected by factors other than behavior. These include genetic, metabolic, and hormonal influences (2–4) that probably predispose some persons to obesity and may well set the range of possible weights that an individual can achieve. Some individuals may never be thin, despite Herculean efforts to modify eating and activity habits. Behavior therapy, however, can help such individuals develop a set of skills (such as eating a low-calorie, low-fat diet) to achieve a healthier weight, even if they cannot attain an ideal one.

Behavioral treatment is based primarily on principles of classical conditioning, which posit that eating is often prompted by antecedent events (ie, cues) that become strongly linked to food intake (1). Behavioral treatment, as described below, helps patients identify cues that trigger inappropriate eating (and activity) and learn new responses to them (5, 6). Treatment also seeks to reinforce (or reward) the adoption of positive behaviors.

In the past 20 y, cognitive therapy also has been incorporated in the behavioral treatment of obesity. The underlying assumption of cognitive therapy is that thoughts (or cognitions) directly affect feelings and behaviors (7). Negative thoughts frequently are associated with negative outcomes, as in the case of an individual who overeats thinks, “I’ve blown my diet,” and then proceeds to eat even more. Cognitive therapy can help people to identify these thinking errors and then to overcome them. Cognitive therapy and its application to weight management are described below.

DEFINING CHARACTERISTICS

Behavioral treatment has several distinguishing characteristics (13). First, it is goal directed. It specifies very clear goals in terms that can be easily measured. This is true whether the goal is walking four times per week, lengthening meal duration by 10 min, or decreasing the number of self-critical comments. Specific goals facilitate a clear assessment of success.

Second, treatment is process oriented. It is more than helping people to decide what to change (ie, eating, activity, and thinking habits); it is helping them identify how to change (8). Thus, once a goal is specified, patients are encouraged to examine factors that will facilitate or hinder goal achievement. In cases in which the desired behavior is not implemented, problem-solving skills are used to identify new strategies to overcome barriers. In this view, successful weight management is based on skills that can be learned and practiced, in the same manner that an individual can learn to play the piano through frequent practice. Skill power, not will power, is the key to success.

Third, the behavioral approach advocates small rather than large changes. This is based on the learning principle of successive approximation in which incremental steps are taken to achieve more distant goals. Making small changes gives patients successful experiences on which to build rather than attempting drastic changes that are typically short-lived.

The behavior change process is facilitated through the use of a variety of problem-solving tools. The behavior chain, an illustration that visually depicts the chain of events that lead to an unwanted behavior such as overeating, is one of the tools commonly used in treatment (Figure 1). By examining the cues and

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events that lead up to an overeating episode, one can identify areas in which modifications in behavior can be made to break the chain of events and prevent an overeating episode from occurring in the future. For example, if a patient has identified television watching as part of the sequence of events leading up to an overeating episode, limiting eating to a more appropriate location (ie, table in the kitchen or dining room) can be an effective strategy for weakening the association between eating and television watching. The more often the patient refrains from eating in front of the television, the less likely that television watching will automatically trigger eating.

THE BEHAVIORAL PACKAGE

Behavioral treatment usually includes multiple components, such as keeping food and activity records (ie, self-monitoring), controlling cues associated with eating (ie, stimulus control), nutrition education, slowing eating, physical activity, problem solving, and cognitive restructuring (ie, cognitive therapy) (5, 6). These components comprise the "behavioral package" that has been summarized in manuals such as the LEARN Program for Weight Management 2000 (6). Studies have shown that two components, self-monitoring (14, 15) and physical activity (16), are consistently associated with better weight control in the short- and long-term, respectively. Additional research is needed to identify the most potent components of the package, as well as additional interventions that might be added (such as body image therapy) to improve efficacy, especially in the long term. In the interim, researchers and practitioners probably will continue to use the behavioral package because it is well validated, as a whole, and different patients are drawn to different components of the intervention.

SHORT-TERM RESULTS OF BEHAVIORAL TREATMENT

A large number of clinical studies have been conducted examining the effects of behavioral treatment on weight loss. The typical design of most behavioral treatment weight loss studies is group meetings weekly for the initial treatment phase (=3-6 mo), biweekly (every other week) meetings for the maintenance phase (6–12 mo), and monthly or bimonthly for the later phases of the study (12–24 mo) (13, 17, 18).

Wing et al (19) reviewed behavioral weight loss studies from 1996 to 1999, which resulted in a mean short-term weight loss of 10.6% (9.6 kg) during the treatment phase (21 wk) and 8.6% (6.0 kg) at follow-up (18 mo). Studies published from 2000–2004 have produced similar results (20–23).

STRATEGIES FOR AUGMENTING OUTCOMES

Although behavioral treatment provides individuals with a set of skills to handle barriers to eating healthy and being active, overcoming barriers is a difficult endeavor in a fast-paced environment that encourages overconsumption of energy-dense, palatable, low-cost foods and promotes energy-saving devices (24, 25). A healthy lifestyle requires significant planning, proficiency in making healthy choices and estimating portion sizes, and diligence in monitoring caloric intake and activity, all of which take time to develop and maintain. As such, strategies for simplifying and making this process more practical have been investigated and are described below. In general, these strategies provide structure and reduce time spent in meal planning and decision making.

Food provision

Jeffery et al (26) examined the impact of food provision on weight loss outcome in 202 overweight individuals. Participants who received food along with standard behavioral treatment lost more weight at 6 mo (−10.1 vs −7.7 kg), 12 mo (−9.1 vs −4.5 kg), and 18 mo (−6.4 vs −4.1 kg) than those who were prescribed a reduced calorie diet and standard behavioral treatment. In a subsequent study, Wing et al (27) sought to determine whether food provision itself or limited dietary decision making affected weight loss outcome. In this study, 163 overweight women were randomly assigned to one of four interventions: 1) standard behavioral treatment, 2) standard behavioral treatment plus written meal plans and grocery lists for five breakfasts and dinners each week, 3) standard behavioral treatment plus foods free of charge, and 4) standard behavioral treatment plus foods free of charge for a charge of $25.00/wk. Data were collected for ~90% of participants at the end of active treatment (ie, 6 mo) and at 1-y follow-up (ie, 18 mo after randomization). Weight loss was greater in groups that received food and meal plans compared with the group that received standard behavioral treatment at 6 and 18 mo; however, no differences in weight loss were observed.
between the groups that were provided food and the group that received meal plans and grocery lists.

Meal replacements

Similar findings are observed in studies that compared meal replacements (28–31) or prepackaged entrees (32, 33) with self-selected diets. These studies suggested that replacing two of three meals with a liquid and/or solid meal replacement or at least two meals with a portion-controlled entrée resulted in greater weight loss than self-selected diets. Although some weight regain was observed over time, a greater reduction in weight was observed even up to 4 y in individuals receiving meal replacements (28). Based on a meta-analysis by Heymsfield et al, (34) individuals consuming meal replacements lose ≈7–8% body weight, whereas those on a standard self-selected diet lose 3–7% body weight at 1 y. It is unclear whether meal replacements are superior to other structured weight loss approaches that provide menus and recipes, however. Noakes et al (35) found similar decreases in weight in individuals using meal replacements (−9.0 kg or −9.4% body weight) and those following structured diets (−9.2 kg or −9.3% body weight) at 6 mo. These findings suggest that increasing structure may improve dietary compliance.

Pharmacotherapy

Another method used to enhance weight loss outcomes is to couple behavioral and pharmacotherapy approaches. It can be argued that behavioral treatment modifies the external environment, whereas pharmacologic approaches modify the internal environment either centrally (eg, sibutramine) or peripherally (eg, olistat). To test this hypothesis, Wadden et al (36) compared weight loss outcome in women randomized to one of three groups: 1) sibutramine alone, 2) sibutramine plus behavior modification in the context of a self-selected reduced calorie diet conventional diet, or 3) sibutramine plus behavior modification in the context of a portion-controlled diet using meal replacements. Because all patients received the same dosage of sibutramine, this study assessed the dose response of increasing behavioral treatment. These investigators found that, at 6 mo, the group with the most behavioral modification lost approximately three times the amount of weight (−17.7% of initial body weight) than those on drug alone (−5.8% of initial body weight) and were better able to maintain the loss at 1 y (−16.5 vs −4.1%, respectively). These data suggest that increasing the “dose” of behavioral therapy will increase both short- and long-term weight loss.

Commercial weight loss programs

Most research on behavior treatment has been conducted in university-based clinic programs. Although such studies are important, they tell us little about the effectiveness of these approaches in settings outside of specialized clinics. Womble et al (37) reviewed the available literature on commercial approaches and found, in general, that weight losses were less than those observed in the clinical setting. In a recent study that evaluated the effectiveness of the Weight Watchers program, Heshka et al (38) found that those in Weight Watchers lost more weight than those assigned to a self-help group (4.6 vs 1.7%) after 1 y and after 2 y (3.1 vs 0.2%).

SETTING THE STAGE

Talking with patients about weight control

No matter what type of obesity treatment is ultimately recommended, effective and compassionate treatment of obese patients requires an understanding of the cultural context in which treatment occurs. As Stunkard and Sobal (39) have suggested, disparagement of obese individuals is the last socially acceptable form of prejudice. It is not surprising, therefore, that health care providers seem to share the negative view of the overweight by society. Studies suggest that a large proportion of physicians consider obesity a behavioral problem (ie, lack of willpower or lack of physical activity) and view obese patients as lazy, awkward, unattractive, and ugly (40, 41). Other health care providers, such as dietitians, also have negative or at best ambivalent attitudes toward the obese (42–44). Such characterizations are likely to lead to behaviors that may be discriminatory. There are numerous clinical anecdotes about how obese patients have been treated disrespectfully in the health care setting.

Toward more empathic encounters

It can be argued that overweight patients are “just too sensitive,” and their perceptions about medical visits reflect their own frustration with their weight rather than any systemic bias by health care professionals. Even if patients’ bad experiences are partly attributable to their inaccurate perceptions, such experiences need to be remedied. This is necessary because these inaccurate perceptions lead to interactions that, at best, provide health care at the expense of a patient’s self-esteem or, at worst, prevent obese patients from seeking health care altogether. The following recommendations seek to put obese patients at ease in the health care setting and promote competent, compassionate care (45).

Assume that obese individuals know they are overweight. If they have not heard it from a health care professional, they have probably been told by friends, family or even strangers. Simple phrases such as, “What do you think about your weight?” will allow you to assess the patient’s interest and/or motivation for weight control in a nonjudgmental manner. They also allow you to hear the patient’s perspective before making any recommendations for weight loss or describing the ill effects of excess weight.

Be empathetic about dissatisfaction with weight and/or shape. It is reassuring for patients to hear from their health care providers things such as “Weight control is really tough work, isn’t it?” or, “It must be frustrating to have worked so hard and still be unhappy because you haven’t lost as much weight as you wanted.” Such phrases let patients know that you understand their difficulties and that you will not be judgmental.

Listen carefully to the patient’s presenting problem, independent of weight. Few patients consider weight to be their primary problem. As Stunkard (45) points out, patients define the presenting problem. If weight is a precipitating condition, focus on the factors that affect the presenting problem and weight. For example, it is not likely to be useful to tell an obese patient with dyslipidemia to lose weight. Encouraging the same patient to decrease the intake of saturated fat and make small changes in activity, however, will likely influence weight and lipids.
Create a user-friendly office

Just as airline seats are frequently too small for significantly obese patients, so are the equipment and furnishings found in many health care settings. Attention to the following details facilitates an environment that is receptive to obese patients (13).

Have a scale that can weigh all patients. Getting weighed is among the most unpleasant experiences for an obese patient in the health care setting; it becomes humiliating if a patient weighs more than the scale can accommodate.

Have gowns available that fit larger patients. Many obese patients report the experience of waiting for a physician examination in a gown that barely covers them.

Use larger blood pressure cuffs when appropriate. Office and/or hospital staff should know when to use larger cuffs with patients. Inappropriate cuff sizes will lead to inaccurate measurements and treatment recommendations. Moreover, having a cuff inflate off a patient’s arm is awkward for both the patient and practitioner.

Provide some armless chairs in the waiting room. Obese patients should not be made to feel uncomfortable in chairs made for lean persons.

Counseling skills

Although behavioral change is the responsibility of the patient, it is the responsibility of the health care provider to facilitate change through effective counseling (46). Counseling is not an innate talent; it requires practice and fine-tuning. Many providers may feel that their primary role is to give advice to patients about healthy methods of weight control. Although some education and advice is useful, most weight control patients are well aware of what they “should” eat; the problem is doing so in an environment that encourages otherwise. Therefore, an emphasis on problem solving (46).

Improving adherence

Several straightforward guidelines can help patients improve their adherence to the behaviors necessary for effective weight control (13, 46). They are discussed below.

Be clear that the patient knows the rationale for changing behavior (WHY). It is important to ensure that patients understand the rationale for a specific behavior change. Statements such as “Why do you think I’m asking you to keep food records? Have you found them useful in the past?” will clarify whether the patients see any value in record keeping. Patients are adult learners who need to be engaged in the process of behavior change. Simply telling them that “it is important” or “good for them” does not suffice.

Identify a goal and establish a specific plan (WHAT). Short-term goals should be based on behavior rather than weight because many factors other than behavior (salt, fluid, and humidity) affect weight in the short term. When helping patients select goals, it is important to describe the behavior in concrete and specific terms: help patients select a specific plan (eg, limit eating to 300 kcal between 7:00 and 10:00 PM or walk three times for 20 min after dinner on Monday, Wednesday, and Friday) rather than a general platitude (eg, eat less at night or exercise more). The more specific the goal, the better.

Identify facilitators and barriers to success (HOW). To successfully execute the plan, the process must be thought through from beginning to end. It is rare that any plan will proceed without an occasional glitch; therefore, it is important to help patients think through the steps that will be necessary to achieve their goal (eg, purchasing alternative foods for evening consumption and having a spouse help with household duties after dinner), including steps to avoid or overcome potential barriers.

Have the patient make a written record of the plan and key steps in its implementation. In addition, make a brief note in the chart documenting the specific plan. At the next visit, review the patient’s progress with the specific plan rather than asking generally, “How did it go?” Was the behavior accomplished or not? If successful, what strategies did the patient use to achieve the goal? If unsuccessful, what things got in the way and how can they be removed in the future? Patients benefit more from examining how behavior changed or did not change rather than focusing on why things did not go as planned.

Dealing with nonadherence

In an ideal world, the steps described above would reliably produce adherence, weight loss, and satisfied patients and practitioners. The reality is that effective weight control is a learned skill. Like the development of any other skill, setbacks are to be expected. Effectively managing nonadherence is an essential skill for both patients and practitioners. The following suggestions are designed to help the health care provider and patient deal with nonadherence (13).

Assume that a lack of planning or skills rather than a lack of motivation is the reason for nonadherence. When things do not go as planned, it is important to focus on what can be done differently in terms of planning or coping skills. This emphasizes a plan of action rather than fruitless discussions about motivation. Motivation (the ratio of benefits to costs) can be addressed after multiple attempts at planning and skill building have faltered, but the first assumption should be that skills, rather than motivation, need to be enhanced.

Analyze what happened and how it can be prevented in the future. Cast setbacks as an opportunity to refine weight control skills. Ask enough questions to understand how things did not go as planned (eg, time, place, activity, emotions, and the sequence of events). What specifically got in the way and how can it be removed? Instilling hope is an essential feature of an effective therapeutic relationship. Having patients think through how they would deal with same situation in the future will increase self-efficacy and give hope.

Help patients recognize nonadherence and assume responsibility for their actions. Patients may attribute nonadherence to factors that are beyond their control (eg, people and activities). Identifying behaviors that can be managed not only empowers the patient, it also underscores that the patient is responsible for problem solving.

Avoid criticizing patients. Weight control is tough work, and patients need to know that you will not give up on them. Criticizing patients or questioning their motivation does little for
improving adherence and has adverse effects on the patient-health care provider relationship. Patients will struggle, and they need to know that your support is unconditional and you will not give up on them.

Preserve the patient’s self-esteem and be patient. Realize that patients often feel frustrated and discouraged when they have not followed their intended plan. Feelings of failure are likely to occur when one expects to achieve perfection. Identifying small and positive accomplishments and pointing out that the goal is not to achieve perfection will help boost morale and self-esteem. Both the health care provider and the patient should keep in mind that long-term changes do not occur overnight.

Share your frustration with colleagues so it does not affect your work with patients. Treating refractory conditions such as obesity can undermine your own professional self-esteem. Read the scientific literature regularly to be reassured that no one has yet cured obesity! Discussing frustrations with colleagues can have positive therapeutic benefits and reduce the potential for burnout and ineffective patient care.

Unrealistic expectations

One of the greatest challenges in the clinical management of obese patients is addressing the significant disparity between actual and expected weight losses. Although professionals generally accept a 10% weight loss as successful (based on the associated improvements in comorbidities), patients typically seek weight losses that approximate 30% reductions in body weight (9, 47–49). Several recommendations may help patients accept more modest weight loss outcomes as successful.

Be clear about what weight loss does and does not. Weight loss will make you healthier, but it does not guarantee a better job, a happier marriage, or other things that many patients seek through weight loss. Discussing (before treatment) what else patients expect to change besides their weight will help identify any unrealistic expectations or magical thinking regarding weight loss.

Focus on nonweight outcomes. Focus on the many nonweight changes, such as improvements in serum lipids, blood pressure, and glycemic control. In addition, prompt patients to assess changes in their quality of life, such as increased energy, being able to keep up with children or grandchildren, and climbing stairs without shortness of breath.

Discuss biological limits. In short, acknowledge what patients already know: not everyone who eats the same and exercises the same weighs the same. Weight is not infinitely malleable, and there are likely biological boundaries that set limits for weight loss. Help patients focus on behavioral changes that improve health and worry less about the ultimate number of pounds lost. Patients will need your help to counter the cultural myth that “you can weigh whatever you want.”

A FINAL NOTE

Health care providers can provide a great service to obese patients by reminding them that their worth is not measured on the scale. Patients should be encouraged to take themselves, their health, and, thus, their weight seriously rather than attempting to lose weight so they can like themselves. Reaffirming the patient’s self-worth, independent of body weight, is perhaps one of the most powerful interventions a health care provider can provide an obese patient. As Stunkard (45) suggests, “As with any chronic illness, we rarely have an opportunity to cure, but we do have an opportunity to treat the patient with respect. Such an experience may be the greatest gift that [we] can give an obese patient.”

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The influence of food portion size and energy density on energy intake: implications for weight management

Julia A Ello-Martin, Jenny H Ledikwe, and Barbara J Rolls

ABSTRACT
The increase in the prevalence of obesity has coincided with an increase in portion sizes of foods both inside and outside the home, suggesting that larger portions may play a role in the obesity epidemic. Although it will be difficult to establish a causal relationship between increasing portion size and obesity, data indicate that portion size does influence energy intake. Several well-controlled, laboratory-based studies have shown that providing older children and adults with larger food portions can lead to significant increases in energy intake. This effect has been demonstrated for snacks and a variety of single meals and shown to persist over a 2-d period. Despite increases in intake, individuals presented with large portions generally do not report or respond to increased levels of fullness, suggesting that hunger and satiety signals are ignored or overridden. One strategy to address the effect of portion size is decreasing the energy density (kilojoules per gram; kilocalories per gram) of foods. Several studies have demonstrated that eating low-energy-dense foods (such as fruits, vegetables, and soups) maintains satiety while reducing energy intake. In a clinical trial, advising individuals to eat portions of low-energy-dense foods was a more successful weight loss strategy than fat reduction coupled with restriction of portion sizes. Eating satisfying portions of low-energy-dense foods can help to enhance satiety and control hunger while restricting energy intake for weight management. Am J Clin Nutr 2005;82(suppl):236S–41S.

KEY WORDS  Portion size, energy density, energy intake, body weight, weight management, obesity

INTRODUCTION
In the United States, portion sizes of many foods have been increasing since the 1970s; (1) this trend has been observed in a variety of settings including restaurants, supermarkets, and in the home (2–4). Increases in portion size have occurred in parallel with the rise in the prevalence of obesity, which suggests that large portion sizes could play a role in the increase in body weight (5). These observational studies should be interpreted with caution, because they cannot demonstrate causality. Investigators have not yet systematically examined the relationship between the portion size of foods and weight status; therefore, a crucial step in assessing this relationship is to determine experimentally whether portion size affects energy intake. Portion size, however, is only one of many factors that may encourage the overconsumption of food. Energy density (kilojoules per gram; kilocalories per gram) has been shown to significantly affect energy intake; foods that are high in energy density increase energy intake while foods that are low in energy density decrease intake (6, 7). Thus, it is important to examine how energy density and portion size interact or combine to affect intake. This review will examine the experimental evidence related to the influence of portion size on energy intake and satiety. The impact that energy density and portion size have on energy intake will also be discussed, along with implications for weight management.

THE EFFECT OF PORTION SIZE ON ENERGY INTAKE
Children and portion size
In very young children, food intake appears to be relatively unaffected by portion size. Nationally representative data indicate that the average portions of foods consumed by 2-y-olds have remained stable over a 20-y period (8), although many commercially available products have increased in portion size during this time (3, 4). Data from a controlled study show that, when 3-y-old children were served different portions of macaroni and cheese on three separate occasions, they consumed similar amounts at each meal (9). This suggests that very young children, rather than responding to food cues such as portion size, are able to self-regulate their intake by responding to physiologic cues for hunger and satiety. As children age, however, it appears that internal cues have less effect on food intake, whereas external factors exert more influence. In the study cited previously, when the different portions of macaroni and cheese were served to 5-y-old children, they consumed significantly more energy as the portion size was increased. This response to portion size occurred although their hunger did not differ at the start of the meals (9). Similarly, Fisher et al (10) found that 4-y-old children ate 25% more when they were served an entrée that was twice the size of an age-appropriate portion. The children who increased their intake the most when served large portions were those who had been identified as more likely to eat in the absence of hunger.
It is not clear why children are more influenced by portion size as they age. Data suggest that early experiences lead to the development of behaviors that shape eating habits. In one experimental study, the 4-y-old children who were rewarded for cleaning their plates increased their energy intake (11). Conversely, the children who were taught to focus on satiety cues, indicated by the fullness in their stomachs, ate an appropriate amount of food. Thus, the response to portion size by children could be a learned behavior that leads to a shift of attention away from internal hunger and satiety cues toward food cues in the external environment. A lack of response to satiety signals may predispose children to overeat in an environment in which large portions of palatable foods are readily available (12, 13). The influence of large portions on intake, however, has been shown to be moderated simply by allowing children to serve themselves. One study demonstrated that children ate 25% less of a large entrée when they decided for themselves how much food to put on their plates compared with when they were served the large portion of the entrée by an adult (10).

Although there is a need for more data, these studies suggest strategies for parents and caretakers that may help children to eat appropriate portions. One approach is for adults to provide children with a variety of nutritious foods and allow children to determine how much they will eat by serving themselves (14). Adults should also encourage children to recognize hunger and fullness cues and to rely on these cues for the initiation and termination of eating; children should not be required to clean their plates nor be rewarded for doing so. As we understand more about eating behavior, it is likely that additional strategies will become available to help children preserve their ability to recognize and respond appropriately to internal signals and to resist environmental influences on intake such as portion size.

Adults and portion size

Considering that children over the age of 3 y consume more food when presented with large portion sizes, it is not surprising that adults have been found to respond in a similar way. Some of the first work characterizing the influence of portion size was conducted using different package sizes in naturalistic settings. Wansink (15) examined the effect of portion size on prospective food usage and found that individuals estimated they would consume more food when presented with larger packages than when presented with smaller packages. For example, when asked to dispense an appropriate amount for two people, women poured significantly more spaghetti and oil from larger food packages than from smaller packages. A similar effect was seen with small candy-coated chocolates (15). Thus, it was shown for a variety of foods that the bigger the package, the more food was served from it. It is critical, however, to show that package size and portion size affect not only how much food people serve but also how much they eat.

To systematically assess the effect of portion size on food intake, Rolls et al (16, 17, 19, 20) conducted a series of experiments, beginning with studies of a single meal (Table 1). In one study, when men and women were served different portions of macaroni and cheese on different occasions, they consumed 30% more energy (676 kJ; 162 kcal) when offered the largest portion (1000 g) than when offered the smallest portion (500 g) (16). In another study, when 6-, 8-, 10-, or 12-inch sandwiches were served on different days, both men and women significantly increased their energy intake as the size of the sandwich increased. When served the 12-inch sandwich, women consumed 31% more energy (665 kJ; 159 kcal) and men consumed 56% more energy (1485 kJ; 355 kcal), than when served the 6-inch sandwich (17). In both of these studies, participants reported similar ratings of hunger and fullness at the end of the meals, despite large differences in intake. It is possible that the larger portions of food influenced subjects’ expectations of the amount they could eat, which in turn affected their ratings of hunger and fullness. These results suggest that, in some circumstances, as portion size is varied, individuals adjust their perception of satiety cues while consuming more food.

The effect of portion size has also been observed in more naturalistic settings. A study in a cafeteria-style restaurant tested whether increasing the portion size of a pasta entrée from 248 g (standard portion,1766 kJ; 422 kcal) to 377 g (large portion, 2647 kJ; 633 kcal), while keeping the price the same, would affect intake (18). Increasing the portion of pasta by 50% was associated with a 43% increase in energy intake (719 kJ; 172 kcal) for the pasta.

Without additional data, it could be argued that the effect of portion size on intake has little effect on body weight, because individuals may compensate for increased food intake by eating less at the following meal. One recent study examined how the portion size of a snack affects the energy intake of both the snack and the subsequent meal (19). On different days, subjects were served different package sizes of potato chips (28, 42, 85, 128, or 170 g) as an afternoon snack and were later served a standard dinner. Both men and women significantly increased their intake of potato chips as the package size increased. For example, when served the 170 g package, women ate 18% more and men ate 37% more than when served the 85 g package. As subjects increased their snack intake with increasing package size, they also reported feeling fuller; however, they did not adjust their intake at the subsequent dinner meal to compensate for the increased energy intake and fullness.

To investigate whether portion size has an impact on intake beyond a single eating occasion, Rolls et al (20) conducted a study in which they increased the portion size of all foods served at meals and snacks over 2 d. It was again found that increasing portion sizes led to significantly increased energy intake. When the portions of all foods were doubled, energy intake on both days increased by a mean of 26% for both women and men [2218 kJ/d (530 kcal/d) for women and 3402 kJ/d (813 kcal/d) for men].

Although subjects reported feeling more full after they consumed the larger portions, they did not compensate for the excess energy eaten over the course of the first day by reducing their intake on the second day. These data demonstrate that the effects of portion size can persist over several days, resulting in substantial increases in food and energy intake. Future studies are needed to determine whether these effects continue over the long term and have an effect on body weight.

The experimental evidence demonstrates that portion size has a significant effect on food intake in adults in the short term. The effect of portion size was seen not only with a variety of foods in single meals (16, 17), entrées in a restaurant (18), and foods over several days (19) (Table 1), with a variety of characteristics. Increases in intake were observed in both men and women across a range of ages, body weights, and psychological factors, such as scores for dietary restraint and depression. It is not clear why individuals consistently increased their intake as portion size

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increased. In the single-meal studies (16, 17), it appeared that subjects were unaware of their extra intake, in that they did not report feeling fuller after eating significantly more food. In the studies that included multiple meals (16, 17), subjects reported that they felt fuller, yet they did not respond by eating less within the meal or at subsequent meals. This suggests that adults ignore or override hunger and satiety signals when presented with large portions of food. It is possible that individuals learn to eat in the absence of hunger as young children and continue with this behavior into adulthood (10, 21).

Further insight into adult eating behavior is provided by recent survey data from the American Institute of Cancer Research (22). In a survey of >1000 adults, 69% indicated that, when dining out, they finish their entrées all or most of the time. Of those adults, 30% reported that they would have been satisfied with a smaller portion. This suggests that, when eating out, many adults ignore satiety signals and eat beyond the point of noticeable fullness. Additionally, many survey respondents (42%) reported that they determine the amount of food to eat according to what they are used to eating. The portion sizes that individuals customarily eat may be related to frequent exposure to large portions over time. Clearly, future studies are needed to determine the reasons that individuals fail to rely on satiety cues and instead respond to external cues in the eating environment, such as portion size.

### ADDRESSING THE PORTION SIZE EFFECT

Strategies for addressing the influence of portion size can be directed toward either the consumer or the eating environment. For consumers, one obvious approach is education about appropriate portion sizes. Previous efforts in training individuals to estimate portions of foods have not been notably successful, however, and much work remains to be done in both research and practice in this area (23). An approach that helps consumers resist the influence of large portions and lose weight is the use of commercially packaged meals that are controlled for portion size and energy content (24–26). Another possible strategy could be directed toward either the consumer or the eating environment. Restaurants and other food providers could improve the food environment by offering a wider range of portion sizes or promoting and discounting the purchase of portion sizes that are reasonable rather than oversized. Reducing the portion sizes of foods, whether as a result of individual action, public education, or commercial initiative, may be an overly simplistic approach to moderating the effect of portion size on food intake. Experimental evidence shows that, in the short term, feelings of satiety and satisfaction are determined by the amount or volume of food consumed, as well as by the energy content (27, 28). At some

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### TABLE 1

<table>
<thead>
<tr>
<th>Investigators</th>
<th>Subjects</th>
<th>Duration of study</th>
<th>Manipulation of portion size (PS) and energy density (ED)</th>
<th>Effect on energy intake</th>
<th>Effect on ratings of hunger and fullness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Portion size studies: single meal</td>
<td>Rolls et al (16)</td>
<td>51 men and women</td>
<td>Lunch 1 d/wk for 4 wk</td>
<td>Macaroni and cheese served in different PS (500, 625, 750, or 1000 g)</td>
<td>Macaroni and cheese intake increased with increasing PS (mean intakes: 2284, 2552, 2728, and 2962 kJ)</td>
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<td></td>
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<td></td>
<td>Submarine-type sandwich served in different PS (6-, 8-, 10-, or 12-in)</td>
<td>Sandwich intake increased with increasing PS (mean intakes: 2406, 2941, 3226, and 3489 kJ)</td>
</tr>
<tr>
<td>Portion size studies: meal-to-meal</td>
<td>Rolls et al (19)</td>
<td>60 men and women</td>
<td>Snack and dinner 1 d/wk for 5 wk</td>
<td>Potato chips served in different PS (28, 42, 85, 128, or 170 g)</td>
<td>Snack intake increased with increasing PS (mean intakes: 577, 820, 1243, 1502, and 1577 kJ)</td>
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<tr>
<td></td>
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<td></td>
<td>Variety of foods served as meals and snacks in different PS (100%, 150%, or 200%)</td>
<td>2-d energy intake increased with increasing PS (mean intakes: 21,644, 25,191, and 27,363 kJ)</td>
</tr>
<tr>
<td>Portion size and energy density studies</td>
<td>Rolls et al (27)</td>
<td>42 women</td>
<td>Salad and pasta lunch 1 d/wk for 7 wk</td>
<td>Required salad varied in PS (150 or 300 g) and ED (1.38, 2.8, 5.6 kJ/g) was followed by a standard pasta dish eaten ad libitum</td>
<td>Meal energy intake (pasta with salad) was minimized with large salad of lowest ED</td>
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<td></td>
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<td></td>
<td></td>
<td>PS and ED independently increase energy intake. Intake significantly greater (925 kJ) when served large portion of high ED compared to small portion of low ED</td>
<td>Hunger and fullness ratings after lunch did not differ</td>
</tr>
</tbody>
</table>
point, therefore, reductions in portion size will lead to hunger and dissatisfaction and may be perceived by consumers as negative and restrictive. More importantly, large portion sizes are not equally likely to cause overconsumption of energy for all types of foods. For these reasons, we need a directed response to the problem of portion size, which takes into account different food types and provides a positive and practical message to consumers. Recent research suggests that decreasing the energy density of foods may be an effective alternative to a general reduction in food portion sizes.

**THE ROLE OF ENERGY DENSITY**

Energy density refers to the amount of energy in a given weight of food (kilojoules per gram or kilocalories per gram). Of the components of food, water decreases energy density by adding weight but not energy, whereas fat increases the energy density of a food to a greater extent than either carbohydrate or protein. Studies that have systematically examined the effects of energy density of the diet have shown that it directly influences energy intake, an effect that is independent of the macronutrient composition of a food. In one experiment, in which energy density was manipulated but portion size was held constant, subjects were served all of their meals for 2 days on three separate occasions. The meals were mixed dishes that were either low (3.5 kJ/g; 0.8 kcal/g), medium (4.4 kJ/g; 1.1 kcal/g), or high (5.6 kJ/g; 1.3 kcal/g) in energy density. Subjects ate similar amounts of food (by weight) over the 2 d in all three conditions, regardless of the variation in energy density. As a result, when subjects were offered the lower-energy-dense foods, their energy intake over the 2 d was ≈30% less than when they were offered the higher-energy-dense foods. Despite the substantial reduction in energy intake, subjects rated themselves equally full and satisfied (29). Several other studies have confirmed that, when individuals consume meals lower in energy density, their daily energy intake is significantly lower than when they consume meals higher in energy density (29–32).

Until recently, studies have examined the effects of energy density and portion size separately from each other and determined their effects on satiety (the processes involved in the termination of a meal) and satiety (the effects of a food or a meal after eating has ended). However, under free-living conditions, individuals have access to foods that vary simultaneously in both portion size and energy density; therefore, it is important to examine how these two factors interact to affect energy intake both within a single meal and on subsequent intake.

Because the energy density of food can have a significant effect on energy intake when portion sizes are similar, it is of interest to determine how the energy density of food influences intake when portion sizes are varied. The energy density of food may interact with or add to the effects of portion size on ad libitum intake. This was tested by serving women a casserole as an entrée in three different portion sizes (500, 700, and 900 g) and at two energy density levels [5.23 kJ/g (1.25 kcal/g) and 7.33 kJ/g (1.75 kcal/g)] (33). Both portion size and energy density had independent effects and therefore combined to effect energy intake. Thus, subjects consumed the most energy when served the largest portion of the higher-energy-dense entrée (2594 kJ; 620 kcal) compared with when served the smallest portion of the lower-energy-dense entrée (1665 kJ; 398 kcal). The ratings for hunger and fullness did not differ between these two conditions, despite a 56% difference in energy intake (Figure 1). Another study in which the additive effects of energy density and portion size were examined over 2 d showed that the effects persist beyond a single meal and that the large portions of higher-energy-dense foods had the greatest impact on energy intake (34). Thus, both in the short-term and over 2 d, the effects of portion size and energy density add together to influence ad libitum intake, or satiation.

For foods that are low in energy density, satisfying portions can be encouraged, because they produce fullness while adding little energy. A recent controlled study showed that consuming a large portion of a food low in energy density can even displace energy intake at the rest of the meal (27). On different days, subjects were required to consume a first course salad, which was varied in portion size (150 and 300 g) and energy density (1.38, 2.80, and 5.56 kJ/g; 0.33, 0.67, and 1.33 kcal/g); the first course was followed by a main course of pasta that was consumed ad libitum. Energy intake for the entire meal was minimized when subjects ate the large portion of the low-energy-dense salad as a first course; furthermore, when the meal included this salad, energy intake was less than when the meal was eaten with no salad at all (Figure 2). Although subjects consumed significantly less energy at the meal with the large low-energy-dense salad, they felt just as full as when they consumed the meals with the other large salads. Consuming a low-energy-dense broth-based...
soup as a first course has also been found to reduce overall meal intake (35). Thus, the experimental evidence shows that using the effects of both the portion size and energy density of foods is effective in maintaining satiety and reducing energy intake in the short term.

These studies indicate that energy density and portion size work together to influence energy intake and satiety (Table 1). The findings suggest several different strategies that can help to lower energy intake and increase satiety. When choosing entrées to be consumed ad libitum, reductions in both energy density and portion size can significantly decrease energy intake while maintaining fullness. Conversely, when choosing a first course, the greatest enhancement of satiety and reduction in overall meal intake was seen with large portions of foods low in energy density, such as salad or soup. Simple strategies to lower the energy density of meals involve reducing fat and adding water-rich foods, such as soups, vegetables, and fruits (36, 37).

**IS IT POSSIBLE TO EAT SATISFYING PORTIONS AND LOSE WEIGHT?**

A crucial question is whether consuming low-energy-dense foods is an effective long-term strategy for controlling hunger while reducing energy intake and thus will result in weight loss. This strategy was tested in a recent clinical trial (38). One group of obese women was counseled to incorporate into their diet satisfying portions of low-energy-dense foods, such as fruits, vegetables, and broth-based soups, and to choose and prepare foods with less fat. A comparison group was counseled to limit the portions of all foods and to reduce their fat intake. After 6 mo, the women who were advised to eat more low-energy-dense foods consumed significantly more servings of low-energy-dense fruits and vegetables than the comparison group and thus decreased the energy density of their diets. These dietary changes were associated with a 40% greater weight loss in the reduced-energy-density group (9.0 kg; 20 lb) than in the comparison group (6.7 kg; 15 lb) after 6 mo. Contrary to standard advice to eat small portions to lose weight, advice to eat satisfying portions of low-energy-dense foods was a more successful strategy for weight loss. Thus, when giving dietary advice for weight management, the emphasis should be on the types of food that can be eaten in satisfying portions instead of on restrictive messages that advocate reducing the portions of all foods. If individuals choose foods that are low in energy density, they will be able to eat their usual amount of food, and this will help to eliminate the sense of deprivation that can accompany energy restriction.

**CONCLUSIONS**

As obesity rates continue to rise, it is important to understand how properties of food such as portion size and energy density influence energy intake and weight status. The available data indicate that the effects of portion size and energy density combine so that large portions of energy-dense foods are particularly likely to stimulate overconsumption of energy. Although individuals respond to large portions of a variety of foods by increasing energy intake, they do not consistently report or respond appropriately to increased feelings of satiety. The abundance of large portions of inexpensive, energy-dense foods in the current eating environment appears to override satiety mechanisms so that individuals consume more energy than is required for physiologic needs.

Multifaceted approaches are needed that will involve changes to the current food environment, educational initiatives, and participation by individuals (5). Food providers can help individuals moderate their intake by offering foods in a variety of portion sizes that are reduced in energy density and that are palatable and economical. Such food modifications may be particularly successful if they involve little change in consumer behavior. For example, people have come to accept current food products, such as yogurt, that have been reduced in fat and energy density. This is because yogurts have been formulated to remain palatable, the changes are subtle, and the price is acceptable. Other popular foods, such as burgers and sandwiches, could also be modified to decrease the energy density by combining a reduction in fat content with the addition of water-rich vegetables (7). This would allow consumers to eat their usual portions but with less energy and fat.

Well-funded initiatives are vital to provide individuals with the knowledge and skills to make necessary changes. Such initiatives should include tools to aid selection of appropriate portions and should provide information about how the energy density of foods affects decisions about portion size. Consuming low-energy-dense foods allows individuals to decrease energy intake while still consuming satisfying portions and maintaining satiety. Large portions of foods low in energy density, such as fruits and vegetables, are not only acceptable but should be encouraged. Public health messages promoting low-energy-dense foods can be an effective strategy to counteract the effect of large portions on intake and can help to enhance satiety and control hunger while restricting energy intake for weight management.

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Protein, body weight, and cardiovascular health¹–⁴

Frank B Hu

ABSTRACT
Widespread popularity of high-protein diets has drawn controversy as well as scientific interest. By reducing intake of carbohydrates and increasing consumption of fats and proteins, such diets are thought to increase satiety, facilitate weight loss, and improve cardiovascular risk factors. In recent years, many randomized controlled studies have compared the effects of higher-protein diets on weight loss and cardiovascular risk factors with those of lower-protein diets. The aim of this review was to provide an overview of experimental and epidemiologic evidence regarding the role of protein in weight loss and cardiovascular risk. Emerging evidence from clinical trials indicates that higher-protein diets increase short-term weight loss and improve blood lipids, but long-term data are lacking. Findings from epidemiologic studies show a significant relationship between increased protein intake and lower risk of hypertension and coronary heart disease. However, different sources of protein appear to have different effects on cardiovascular disease. Although optimal amounts and sources of protein cannot be determined at this time, evidence suggests a potential benefit of partially replacing refined carbohydrates with protein sources low in saturated fats. Am J Clin Nutr 2005;82(suppl):242S–7S.

KEY WORDS
High-protein diets, weight loss, cardiovascular risk factors, coronary heart disease, low-carbohydrate diets, blood lipids

INTRODUCTION
Today, ≈66% of Americans are overweight, and 33% are considered clinically obese (body mass index ≥30 kg/m²) (1). The rapid increase in the prevalence of obesity, combined with a lack of effective dietary and pharmacologic treatments, has fueled demand for alternative dietary approaches, in particular, high-protein diets. In recent years, high-protein diets gained widespread popularity before scientific evidence on their safety or efficacy. Advocates of such diets often recommend protein intakes at or above 25% of total energy, amounts that are substantially higher than the average consumption of protein in the US diet.

Proponents of several popular diets (especially the Atkins Diet) have long claimed that higher amounts of dietary protein not only facilitate weight loss but also improve cardiovascular risk factors. In recent years, more than a dozen clinical trials have examined the effects of higher-protein diets on weight loss compared with diets lower in protein (2–16). Epidemiologic studies have also assessed the effects of dietary protein on blood pressure and cardiovascular disease. In this review, we provide an overview of experimental and epidemiologic evidence on the role of protein in weight loss and risk of cardiovascular disease.

PROTEIN AND BODY WEIGHT
Recently, Halton and Hu (17) reviewed 15 randomized controlled studies of a higher-protein diet compared with a lower-protein diet on weight loss, which lasted for between 7 d and 1 y and used a wide variety of macronutrient ratios and methodologic designs (2–16). Seven of these investigations found a statistically significant decrease in total body weight for the higher-protein diets (5, 6, 8, 12, 13, 15, 16). Samaha (12) randomized 64 obese patients to receive counseling on maintaining a high-protein, low-carbohydrate diet (22% protein) and 68 to receive counseling on consuming a low-fat, high-carbohydrate diet (16% protein). After 6 mo, the high-protein, low-carbohydrate group lost significantly more weight (5.8 vs 1.9 kg; P = 0.002). However, at 12 mo, weight loss was not significantly different between the two groups (18). Skov (13) found that obese subjects randomized to a high-protein intake (25% of energy) lost significantly more weight (8.8 vs 5.1 kg) and fat (7.6 vs 4.3 kg) after 6 mo compared with those on a low-protein diet (12% of energy). Brehm (6) conducted a randomized trial on 42 obese females. Twenty received a low-fat dietary regimen (17% protein), whereas 22 received a low-carbohydrate diet (23% protein). After 6 mo, the higher-protein group had lost significantly more weight (8.5 vs 3.9 kg; P < 0.01). Yancy (16) conducted a trial on 119 overweight men and women for 6 mo. Those consuming a higher-protein, low-carbohydrate diet (26% protein) lost significantly more weight than those consuming a lower-protein diet (19%) (12.9% of body weight vs 6.7% of body weight; P < 0.001). Foster (8) randomized 33 obese patients to receive counseling on following the Atkins diet and 30 to receive counseling on a conventional low-fat diet (15% protein). The Atkins group lost significantly more weight at 3 and 6 mo but not at 1 y.

Shorter studies with smaller sample sizes have produced mixed results. Baba (5) conducted a 4 wk randomized trial on 13 obese male subjects with hyperinsulinemia. Seven men received

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PROTEIN AND CARDIOVASCULAR HEALTH

Displacement of carbohydrates, especially refined carbohydrates, by dietary protein may also contribute to greater short-term weight loss seen in high-protein diets. Emerging evidence suggests that high glycemic, refined carbohydrates decrease satiety and increase subsequent energy intake (22). In a recent study, Pereira et al (23) found that resting energy expenditure decreased less with a hypocaloric low-glycemic load diet than with an isocaloric low-fat high-glycemic load diet, after a10% weight loss in both diets. These findings suggest that reducing glycemic load may be useful in weight maintenance.

PROTEIN AND BLOOD LIPIDS

Experimental studies in rabbits or rat suggested that cholesterol-free, purified diets containing proteins of animal rather than vegetable sources were hypercholesterolemic and atherogenic (24). However, this cholesterol-raising effect was not observed in other species of animals (eg, pigs) or humans (25, 26). On the contrary, exchanging animal protein for carbohydrates in human diets significantly reduced LDL cholesterol and triacylglycerol levels and increased HDL cholesterol levels (27).

In a crossover study (28), subjects with moderate hypercholesterolemia were randomly assigned to either high-protein (23% energy) and low-carbohydrate (53% energy) diet or low-protein (11% energy) and high-carbohydrate (65% energy) diet for 4–5 wk. Intakes of dietary fat, cholesterol, and fiber were kept constant. The main sources of protein were turkey, cottage cheese, beef, fish, and ham. Exchanging protein for carbohydrate significantly reduced LDL cholesterol (by 6.4%) and triacylglycerol (by 23%) levels and increased HDL (by 12%). Favorable effects on plasma lipids of substitution of protein for carbohydrates were also observed among subjects with familial hypercholesterolemia (29) and normolipidemia (30).

Jenkins (31) conducted a 1 mo study of a high-wheat protein diet (27% energy from protein) compared with a control diet (16% energy protein) and found significant decreases in triacylglycerol and oxidized LDL cholesterol on the higher-protein diet. A recent study by Samaha (12) compared a low-carbohydrate, high-protein Atkins diet (22% energy from protein) with a low-fat diet (16% energy from protein) on severely obese subjects. The higher-protein group had significantly lower triacylglycerols compared with the lower-protein group (−20 vs −4%; P = 0.001) at 6 mo. However, the higher-protein group lost more weight, and this may have accounted for these differences. At 12 mo, although weight loss was no longer statistically different between the two groups, participants in the higher-protein and low-carbohydrate diet experienced great reduction in triacylglycerol levels but less decline in HDL cholesterol levels compared with the low-fat group (18). In addition, Farnsworth (7) and Skov (13) found a significant decrease in triacylglycerols with higher-protein diets, whereas Parker (10) found a significantly lower LDL cholesterol level with a higher-protein diet.

It is known that low-fat, high-carbohydrate diets reduce LDL if substituted for saturated or trans fats, but these diets also reduce HDL levels and raise fasting triacylglycerols (32). However, the observed apparent benefit of exchanging protein for carbohydrate cannot be simply attributed to displacement of carbohydrate in the diet because LDL cholesterol levels were also reduced in metabolic studies. A high-protein diet may decrease triacylglycerol secretion by hepatocytes (33). Rats fed with a

FIGURE 1. Potential mechanisms for higher-protein diets and weight loss.
protein-deficient diet (8% energy) had slight hypercholesterolemia and increased activity of liver β-hydroxy-β-methylglutaryl coenzyme A reductase compared with controls (16% casein diet) (34). These rats also exhibited increased susceptibility of lipoprotein to peroxidation.

PROTEIN AND BLOOD PRESSURE

Higher consumption of dietary protein has been inversely associated with blood pressure in several observational studies and animal experiments (35). Liu et al (36) conducted a meta-analysis of nine cross-sectional studies and found significant inverse associations between dietary protein and systolic and diastolic blood pressure in both men and women. Only two longitudinal studies examined the associations between dietary protein intake and blood pressure. In the Coronary Artery Risk Development in Young Adults study (37), higher protein intake was inversely associated with changes in systolic and diastolic blood pressure in multiethnic groups during 7 y of follow-up. In a 3-y follow-up study of the 662 participants in the Dietary Intervention Study in Children (38), protein intake was significantly associated with lower blood pressure in separate analyses of each nutrient, but the association became nonsignificant when other nutrients were adjusted for in the models. In the Dietary Approach to Stop Hypertension trial (39), whereas fruits and vegetables independently lowered blood pressure, the combination diet with relatively high-protein intake (18% energy) and increased intakes of fruits and vegetables lowered blood pressure even further. However, because the intervention altered overall eating patterns instead of single nutrients, it is not possible to disentangle the effects of protein from those of other nutrients.

PROTEIN AND CORONARY HEART DISEASE (CHD)

Ecological studies suggest a positive association of CHD mortality rates with animal protein consumption ($r = 0.78$) and an inverse association with vegetable protein consumption ($r = -0.40$) (40). This result should be interpreted with caution because the countries with a higher protein intake also have higher intakes of saturated fat and cholesterol and lower intake of fiber. Previous prospective cohort studies have primarily focused on dietary fats, and only a few examined the association with protein intake. A significant positive association between protein intake and CHD was seen in one study (41), but this was not adjusted for intake of specific types of fat. Hu et al (42) specifically examined the relationship between protein intake and risk of CHD in the Nurses’ Health Study. After controlling for age, smoking, total energy intake, percentages of energy from specific types of fat, and other coronary risk factors, the relative risk (RR) of CHD comparing highest quintile (median, 24% energy) with lowest quintile (median, 15% energy) of total protein intake was 0.74 (95% CI, 0.59-0.94).

Recently, Kelemen et al (43) reported that a higher consumption of vegetable protein was associated with a significantly decreased CHD mortality compared with equivalent amount of energy from carbohydrates or animal protein in the Iowa Women’s Health Study.

PROTEIN AND STROKE

Mortality rates from cerebrovascular disease (stroke) are much higher in some Asian countries (including Japan and China) than in countries in North America or Europe. Data suggest that some aspects of the Asian diet (eg, very low animal fat and protein and relatively high salt) may be associated with high rates of stroke (especially hemorrhagic) in some Asian countries (44).

A significant decline in the incidence of stroke in Japan in the past decades has been attributed to increased consumption of animal products, including meat, eggs, and dairy, as well as improved pharmacologic treatment of hypertension (45). A similar decreasing trend in stroke rate, accompanied by increased consumption of animal products, has also been observed in China (46).

Prospective investigation of protein consumption and stroke is limited. During 14-y follow-up of the women in the Nurses’ Health Study cohort, Iso et al (47) found that animal protein intake was inversely associated with risk of intraparenchymal hemorrhage (RR in the highest vs lowest quintiles, 0.32; 95% CI, 0.10–1.00; $P = 0.04$). Lower consumption of saturated fat was associated with increased risk of hemorrhagic stroke, especially among hypertensive women. These data may help explain a heightened risk of hemorrhagic stroke in populations (eg, Asians) with very low consumption of animal fat and protein and very high intake of carbohydrates.

HIGH-PROTEIN FOODS AND CARDIOVASCULAR DISEASE

High-protein animal products (eg, meat, high-fat dairy, and eggs) are also primary sources of saturated fat and cholesterol. Positive relationships between saturated fat, cholesterol, and CHD suggest that regular intake of foods high in saturated fat and cholesterol (eg, red meat and eggs) may increase risk of CHD. However, epidemiologic studies suggest that the effects of protein-rich foods on cardiovascular risk are not entirely driven by saturated fat and cholesterol.

Red meats

There are few data on the direct relationship between intake of red meat and risk of CHD. In a study of California Seventh-Day Adventists (48), higher beef consumption was significantly associated with increased risk of fatal ischemic heart disease in men but not in women. In a case-control study conducted in Italy (49), higher meat and butter consumption was associated with increased risk of myocardial infarction in women. In the Nurses’ Health Study (50), after adjustment for age, consumption of red meat and high-fat dairy products was associated with increased risk of CHD, whereas consumption of poultry/fish and low-fat dairy products was associated with a lower risk. These associations were substantially attenuated in multivariate analyses and became nonsignificant, but the directions of the associations remained unchanged. The ratio of red meat to poultry/fish consumption was more strongly associated with the risk [multivariate RR across increasing quintiles of the ratio were 1.0, 1.00, 1.13, 1.20, and 1.32 (1.07–1.62); $P$ for trend $= 0.001$]. In the Iowa Women’s Health Study, higher consumption of red meat was significantly associated with increased CHD mortality (43).

Frequent consumption of processed meat has been consistently shown to increase the risk of diabetes in prospective studies (51–53). Although processed meats are a major component of the so-called “Western” diet pattern in these study populations, these associations have been found to be independent of the
Western pattern (51, 53). Furthermore, although the associations between red meat consumption and diabetes risk were attenuated by controlling for saturated fat intake, processed meats remained significantly associated with risk (53), suggesting that constituents of processed meat other than fatty acids (eg, nitrite or advanced glycation end products) may play a role in the development of diabetes.

Heme iron, present in high amounts in red and processed meats, may also link intake of processed meats to risk of diabetes. Heme iron is readily absorbed in the gut and contributes significantly to body iron stores. A recent study (54) found that in healthy women, higher iron stores (reflected by an elevated ferritin concentration and a lower ratio of transferrin receptors to ferritin) were associated with an increased risk of type 2 diabetes; this relationship was independent of known risk factors for diabetes.

**Poultry and fish**

A recent meta-analysis indicates that frequent intake of fish is associated with reduced risk of coronary death (55). Other recent data link increased consumption of fish with decreased risk of ischemic stroke (56). Similarly, Hu et al (50) found that exchange of poultry or fish for red meat was associated with a significantly decreased risk of CHD.

Compared with red meat, white meat from chicken and fish contains similar amounts of protein but substantially less saturated fat and cholesterol. Although the protection against CHD afforded by fish is most likely attributable to the anti-arrhythmic effect of omega-3 fatty acids, other nutrients, including protein, might also have beneficial effects.

**Eggs**

There is little direct evidence linking egg consumption to increased risk of CHD. In the Nurses’ Health Study and Health Professionals’ Follow-up Study, moderate egg consumption (up to one egg per day) was not significantly associated with risk of either CHD or stroke (57). It is conceivable that beneficial effects of protein and other nutrients (including protein, B vitamins, and minerals) may have counterbalanced the small adverse effects of cholesterol in eggs. However, among diabetic patients, even moderate consumption of eggs and cholesterol was associated with significantly increased CHD risk (58). Insulin resistance and dyslipidemia may magnify the adverse effects of cholesterol in those with diabetes.

**Dairy products**

Cross-sectional studies and small clinical trials have suggested an inverse association between dairy consumption and body weight, hypertension, and insulin resistance syndrome (59, 60), but data from large prospective studies are limited. Recently, the Coronary Artery Risk Development in Young Adults study reported a strong inverse association between dairy consumption and insulin resistance syndrome among young obese adults (61). In the Health Professionals’ Follow-up, we found a modest inverse association between dairy consumption, primarily low-fat dairy, and risk of type 2 diabetes (62). Few epidemiologic studies have examined the relationship between dairy consumption and risk of CHD. In the Nurses’ Health Study (50), the ratio of high-fat dairy to low-fat dairy product consumption was positively associated with the risk of CHD [multivariate RRs across increasing quintiles of the ratio were 1.0, 0.87, 0.94, 1.17, 1.27 (1.04–1.54); P for trend = 0.0004]. Among the dairy products, whole milk consumption was associated with significantly increased risk, whereas greater consumption of skim milk was associated with a nonsignificantly lower risk. These results suggest potential benefits of substituting low-fat for high-fat dairy products in preventing CHD.

**Nuts**

Nuts are high in monounsaturated and polyunsaturated fats. Most nuts are rich in protein, especially arginine, which is the precursor of endothelium-derived relaxing factor, nitric oxide (NO). NO is a potent vasodilator that can inhibit platelet adhesion and aggregation. It has been suggested that the anti-atherogenic effect of nuts might be related, in part, to the arginine-NO pathway (63). Nuts also contain high amounts of magnesium, copper, folic acid, potassium, fiber, and vitamin E.

Several epidemiologic studies suggest a relationship between intake of nuts and protection against CHD (64, 65). Bazzano et al (66) found a significant inverse relationship between legume consumption (including peanuts) and risk of CHD. Data also show a significant association between higher consumption of nuts and peanut butter and lower risk of type 2 diabetes in women (67). Wien et al (68) have shown that the substitution of nuts for carbohydrates improves insulin sensitivity and blood lipids and facilitates weight loss.

**Soy**

A meta-analysis of 38 controlled feeding studies in humans suggested that substitution of soy protein for animal protein significantly decreased total and LDL cholesterol levels (69). However, the effects of soy protein and isoflavones on blood cholesterol in humans are highly variable, and overall effects appear to be modest (70).

Because soy protein, the usual experimental source of vegetable protein, is not commonly consumed in Western populations, it is difficult to examine the effect of this specific protein on CHD in the United States. In a prospective study of ≈65 000 Chinese women aged 40–70 y old in the Shanghai Women’s Health Study (71), there was a monotonic inverse relationship between soy food intake and risk of CHD (P for trend = 0.003), with an adjusted RR of 0.25 (95% CI, 0.10–0.63) observed for women in the highest versus the lowest quartile of total soy protein intake. Because of its small number of cases (62 CHD cases) and relatively short follow-up (2.5 y), these results need to be confirmed with additional follow-up and larger studies.

**SUMMARY AND CONCLUSIONS**

Experimental data indicate that high-protein diets produce greater short-term (within 6 mo) weight loss; most studies, however, have been small and inconclusive. Clinical trials suggest that the exchange of protein (either animal or plant) for carbohydrates improves blood lipid profiles. Epidemiologic studies have linked high-protein intake with lower risk of hypertension and CHD. In addition, very low levels of animal protein intake have been associated with a significantly increased risk of hemorrhagic stroke.

Studies suggest that different sources of protein have different effects on cardiovascular disease. The effects of white meat from poultry and fish are known to differ from those of red meat from...
beef and pork. Diets containing substantial amounts of red meat, and products made from these meats, appear to increase risk of CHD. Data indicate that substitution of white meat (poultry and fish) for red meat provides health benefits. In addition, consumption of animal products may have opposing effects on CHD and hemorrhagic stroke. Thus, dietary recommendations should emphasize both the amount and sources of protein. One should distinguish poultry and fish from beef and pork. Also, eggs and dairy products should be distinguished from meats. There is little evidence that moderate consumption of eggs has material adverse effects on chronic diseases, and moderate consumption of dairy products may have complex effects, including benefits and risks. In countries where hemorrhagic stroke rates are substantially elevated, very low consumption of animal products may not be optimal. In many situations, the partial displacement of the carbohydrate staple source of energy, such as grain products with animal products, may have neutral or beneficial health effect. However, the use of plant source of protein and fat, such as nuts, legumes, soy, and vegetable oils, may provide even greater health benefits and should therefore be considered simultaneously (72).

In conclusion, emerging evidence from clinical trials indicates that higher-protein diets increase short-term weight loss and improve blood lipids, but long-term data are lacking. Findings from epidemiologic studies show an inverse relationship between increased protein intake and lower risk of hypertension and CHD. However, different sources of protein appear to have different effects on cardiovascular disease. Although optimal amounts of protein cannot be determined at this time, evidence suggests that it may be beneficial to partially replace refined carbohydrates with protein sources low in saturated fats.

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Solutions in weight control: lessons from gastric surgery1–4

George L Blackburn

ABSTRACT
Surgical therapy is currently the only proven way to achieve significant long-term weight loss, improve obesity-related comorbidities, reduce the risk of premature death, and improve quality of life in a large proportion of treated individuals. Roux-en-Y gastric bypass, the most widely performed procedure in the United States, is known to achieve permanent (>14 y of follow-up) and significant (>50% of excess body weight) weight loss in >90% of patients who undergo the operation. Gastric bypass procedures induce physiologic and neuroendocrine changes that appear to affect the weight regulatory centers in the brain. Researchers have begun to explore the molecular pathways responsible for these outcomes. Identifying the differences between surgical and nonsurgical treatments will eventually lead to new therapeutic options. Am J Clin Nutr 2005; 82(suppl):248S–52S.

KEY WORDS Weight loss surgery, WLS, bariatric surgery, gastric bypass, gastroplasty, laparoscopic adjustable gastric banding

INTRODUCTION
Available weight loss treatments for obesity range from diet, exercise, behavioral modification, and pharmacotherapy to surgery, with varying risks and efficacies. Nonsurgical modalities, although less invasive, typically achieve only relatively short-term and limited weight loss in most patients. However, these therapies are very useful in the preoperative period to reduce risks related to surgical treatment of severe (class III) obesity (1).

Weight loss surgery is an appropriate treatment for patients with class III obesity or class II obesity and major comorbidities (2). Data indicate that weight loss surgery is safe and effective, with well-defined risks (1, 3–10), that it is the most effective in terms of extent and duration of weight reduction in selected patients with acceptable operative risks (11). A recent study of >1000 gastric bypass surgery (GBP) patients showed that, after 5 y, there was an 89% reduction in mortality in severely obese patients who had weight loss surgery compared with those who did not (3). A new meta-analysis indicates that weight loss surgery is one of the most effective treatments for diabetes, hypertension, obstructive sleep apnea, and high cholesterol in severely obese patients (12).

The rapid spread of severe obesity, combined with lack of adequately effective dietary and pharmacologic treatments, has led to growing demand for weight loss surgery. Between the early 1990s and 2003, the number of procedures performed nationwide rose from around 16 000 to more than 100 000 per year. Continued growth is expected, with >140 000 procedures anticipated for 2004 (Figure 1) (5). However, fast growth in an unregulated environment has raised concerns that obesity surgery is being performed by those who have inadequate training and experience or are practicing in hospitals and clinics with inadequate facilities and personnel. Those concerns are being addressed (13).

MAJOR MECHANISMS
Surgical treatment produces weight loss via two major mechanisms: gastric restriction and intestinal malabsorption (Table 1). Restrictive operations involve creation of a small neogastric pouch and gastric outlet to decrease food intake. Examples include vertical banded gastroplasty (VBG) (Figure 2A) and laparoscopic adjustable gastric banding (LAGB) (Figure 2B). Malabsorptive procedures involve rearrangement of the small intestine to decrease the functional length or efficiency of the intestinal mucosa for nutrient absorption. Examples include jejunoileal bypass (Figure 3A), biliopancreatic diversion (Figure 3B), and duodenal switch (Figure 3C). Malabsorptive surgeries produce more rapid and profound weight loss than restrictive procedures but put patients at risk for such metabolic complications as vitamin deficiencies and protein energy malnutrition. Restrictive procedures are considered simpler and safer than their malabsorptive counterparts but tend to result in inferior long-term weight loss.

TYPES OF SURGERIES
Accrued clinical experience and advances in technology have shaped and changed the field of weight loss surgery. Some procedures have evolved, whereas others have become obsolete. Over the past decade, VBG has been replaced by Roux-en-Y gastric bypass (RYGB) and newer laparoscopic approaches. The LAGB, introduced to the US market in 2001, has become increasingly popular. Surgeries currently being performed include gastric bypass, malabsorptive procedures (eg, biliopancreatic diversions), and restrictive operations (gastroplasties with the use of adjustable

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gastric bands). RYGB and LAGB are the most common weight loss surgeries in the United States. Each can be performed either laparoscopically or in an open manner. Biliopancreatic diversion with duodenal switch, although effective in producing weight loss, is still considered investigational by many surgeons because of limited data on long-term safety and metabolic side effects. Others, who have developed particular expertise with the procedure, consider it an important approach to management of extreme obesity.

ROUX-EN-Y GASTRIC BYPASS

RYGB is the gold standard procedure for weight loss surgery in the United States today and the most frequently performed operation. It produces greater long-term weight loss than gastric partitioning alone or VBG. It is also substantially safer than jejunoileal bypass. The most important feature of RYGB (Figure 2C) is a small neogastric pouch and a tight stoma that limits oral intake, making restriction the primary mechanism for weight loss.

The procedure involves creating the small stomach pouch and rerouting a portion of the alimentary tract to bypass the distal stomach and proximal small bowel. This process leads to significant long-term weight loss and improvement or resolution of obesity-related comorbidities (12). Long limb (>150 cm) RYGB may produce superior short-term weight loss in patients who are >200 lb/91 kg overweight or have body mass index ≥50. Optimal limb length is unknown, but long-term follow-up indicates that the benefit of longer limb length decreases over time and may disappear completely.

RYGB is not without risks. These include the following: infrequent but serious surgical complications, eg, pulmonary embolism, intestinal leak, wound infection, and staple line failure; long-term deficiencies of iron, calcium, vitamin B12, and vitamin D; and the possibility of weight regain. Benefits have been found to outweigh these risks (12).

LAPAROSCOPIC WEIGHT LOSS SURGERY

Weight loss surgeons have developed laparoscopic approaches to gastric bypass and other weight loss surgery procedures. Like open procedures, laparoscopic weight loss surgery has proven effective at producing significant and sustained weight loss, along with improvements in comorbid conditions and quality of life. Because it is less invasive than open surgery, it shortens recovery time (14). Additional benefits include decreased rates of wound infection and incisional hernia (14–16). Laparoscopic surgeons gain access to the abdomen via several small incisions. They insert a tiny video camera through one of the incisions and surgical instruments through the others. They
operate by watching their work on a large-screen monitor. Laparoscopic techniques for weight loss surgery are difficult and associated with a longer and steeper learning curve than equivalent open procedures.

Open and laparoscopic RYGB produce similar short-term weight loss and improvements in comorbid medical conditions. The laparoscopic approach improves short-term recovery from surgery and has a lower incidence of incisional hernias than the open RYGB; long-term data are not yet available. Laparoscopic RYGB, although increasingly common, needs to be performed by appropriately trained and qualified laparoscopic weight loss surgeons.

LAPAROSCOPIC ADJUSTABLE GASTRIC BANDING

In LAGB, an adjustable silicone band is placed around the upper stomach to create a small pouch and a restricted outlet. The diameter of the outlet can be changed by injecting or removing saline through a portal under the skin. If the device is ineffective, or if serious complications develop, the band can be removed.

Although a large body of evidence, especially from European studies, suggests that LAGB is effective and safe for weight loss, long-term data from US patients are still limited. Studies indicate variation in benefits, the source of which remains unclear. Complications from gastric banding include band migration or erosion, gastroesophageal reflux disease, esophagitis, and problems with the subcutaneous port or tubing.

LAGB produces variable short-term weight loss and improvements in obesity-related comorbidities, with lower average mortality rates than RYGB or malabsorptive procedures. Placement of the LABG in the pars flaccida path rather than the retrogastric position appears to reduce the incidence of postoperative complications.

EFFICACY OF WEIGHT LOSS SURGERY

GBP has the most profound effect on reward-based eating, suggesting alteration of the CNS “reward pathways.” It decreases the intensity of hunger and enhances the effectiveness of satiety to decrease food intake. GBP also dramatically alters food preferences and selection independent of specific cravings or aversions. The exact mechanisms for these outcomes have yet to be identified.

Neuroendocrine changes are thought to be key factors in producing weight loss. Gastrointestinal regulators of energy balance include those that promote energy storage [ie, ghrelin, glucose-dependent insulinoitropic polypeptide (GIP), galanin, bombesin, and glucagon] and those that promote energy dissipation (ie, peptide Y Y 3-36, glucagon-like peptide-1, oxyntomodulin, pancreatic polypeptide, urocortins, cholcecytokinin, and insulin). Ghrelin, the 28 amino acid neuroendocrine peptide secreted by the stomach, is the most potent endocrine stimulator of appetite and food intake. Ghrelin has been identified as the natural endogenous ligand for the growth hormone secretagogue receptor (17). It is thought that there may be ghrelin receptors on hypothalamic neurons central to weight regulation.

Weight loss through nonsurgical means increases circulating ghrelin. Plasma ghrelin levels are low after gastric bypass (18–21), although not all studies agree (22, 23). GIP secreted from the duodenum and jejunum is thought to promote fat synthesis and deposition. Whereas absence of GIP signaling appears to protect against obesity, secretion has been found to be acutely stimulated by food intake. GIP response to a meal increases after diet-induced weight loss. Surgery is fundamentally different from dieting. It changes the physiology to reset energy equilibrium, it affects the complex weight regulatory system at multiple levels, and it inhibits environmental influences on weight regulation and defeats powerful mechanisms that are inappropriately active in obesity.

Cummings et al. (24) suggest that the RYGB mediates weight loss and improved glucose tolerance via mechanisms that include following: 1) gastric restriction, which limits energy intake; 2) bypass of the foregut, which impairs ghrelin secretion in the long-limb variants of RYGB; and 3) expedited delivery of nutrients to the hindgut, which enhances the ileal brake and stimulates the release of peptide YY and glucagon-like peptide-1. In some patients, a dumping reaction to ingestion of concentrated carbohydrates may contribute to weight loss.

In addition to promoting weight loss, weight loss surgery is known to improve or resolve hyperlipidemia, diabetes, obstructive sleep apnea, and hypertension (12). Resolution of diabetes has often been found to occur within days of weight loss surgery.
before marked weight loss (25). This effect was more prevalent after the predominantly malabsorptive procedures (bileopancreatic diversion or duodenal switch) and the mixed malabsorptive/restrictive gastric bypass compared with the purely restrictive gastroplasty and gastric banding procedures (12).

The putative extent and time relations of the different operative procedures to diabetes resolution may be related to some of the changes in the gut-related hormones. The hormonal milieu, or the relative balance of foregut mediators, is differently affected when the distal stomach is bypassed or a partial gastrectomy is performed, and the enteric contents are separated from the bilipancreatic stream in the upper small intestinal tract (12).

Current metabolic studies of patients with diabetes undergoing weight loss surgery have shown the following: a recovery of acute insulin response (26); significant decreases of inflammatory indicators (C-reactive protein and interleukin) (27); improvement in insulin sensitivity correlated with increases in plasma adiponectin (28, 29); significant changes in the entero-glucagon response to glucose (30); significant reduction in ghrelin levels after gastric bypass (19) but not gastric banding (31); and significant improvement in β cell function after gastric banding (32).

CONCLUSIONS

Surgical therapy is the most effective modality for treatment of severe obesity (1, 3, 4, 12). The most commonly performed procedure, gastric bypass, causes changes in circulating ghrelin and GIP levels that promote weight loss observed after gastric bypass surgery. The reduction in ghrelin and GIP levels are opposite to the increases caused by diet-induced weight loss. These changes are thought to contribute to the greater efficacy of gastric bypass. Other neuroendocrine mechanisms are likely to be involved as well.

Changes in gut-related hormones are believed to be involved in both weight loss and improvement or resolution of obesity-related comorbidities. Improvement or resolution of diabetes has been found to occur within days of weight loss surgery, before marked weight loss (12). The study of the impact of the various weight loss surgical procedures on leptin, ghrelin, resistin, acylation-stimulating protein, adiponectin, entero-glucagon, cholecystokinin, and other gastrointestinal satiety mediators is receiving increasing attention (22, 28, 33, 34).

The field of weight loss surgery is changing at an accelerating rate, one that makes it incumbent on leaders in the field to establish best practice standards for weight loss surgery. Patients will be well served by the development of standards and systems that will make it easier to track high incidences of complications or mortality. The field of weight loss surgery will be well served by future research that focuses on standardizing the technical aspects of weight loss surgery and comparing the efficacy and safety of malabsorptive and restrictive procedures (13).

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Now and again: the food and beverage industry demonstrates its commitment to a healthy America

Susan Finn

ABSTRACT
There exists a complex relationship between food and health in our society that is intrinsically linked to our obesity epidemic. The food and beverage industry recognizes that it can influence and modify the eating behavior of Americans. The American Council for Fitness and Nutrition was formed in January 2003 as a partnership of food and beverage companies, trade associations, and nutrition advocates that work together to create long-lasting remedies for the obesity epidemic. The American Council for Fitness and Nutrition recognizes that the current American lifestyle contributes to an energy imbalance and, therefore, supports approaches that aim to correct that imbalance. The American Council for Fitness and Nutrition also supports the underrepresented populations that are disproportionately affected by obesity, specifically, the African American and Hispanic American communities. Cooperation between industry, government, and academia will be key in establishing long-term strategies to help consumers make healthy lifestyle choices. Am J Clin Nutr 2005;82(suppl):253S–5S.

KEY WORDS Food and beverage industry, healthy America, energy balance, food and health, healthy products, consumer choices

INTRODUCTION
The escalating incidence of overweight and obesity among Americans, adults and children alike, has catapulted scrutiny of the food–health dynamic into the national spotlight. Everyone has an opinion on the issue, from public health advocates and policy makers to food and beverage companies and consumers. Who is responsible for ensuring that Americans of all ages follow a healthy diet? Government? Scientists? Food makers and marketers? Schools? Parents? The answer is all of the above.

As in any multifaceted issue, perspective is everything. The complex relationship between food and health is no different. Where some observers see dichotomy, others see synergy. Everyone can agree, however, that the challenges surrounding food and health cannot be overcome unilaterally. Indeed, everyone with a stake in the issue has something to bring to the table. It is in these divergent views that we find the fodder to create sustainable solutions.

Today, our most pressing food and health issue is obesity. In fact, obesity is altering the way we view our behavior, our beliefs, our culture, and our laws. The nutrition and behavioral research we are conducting and the participatory models that we are creating to halt the obesity epidemic are laying the foundation for how we will address other food- and health-related challenges in the future. Rest assured, such challenges will arise. The die has been cast: we can no longer separate how we eat from how healthy we are, or are not.

A CLOSER LOOK AT OBESITY
Like many public health crises, obesity did not happen overnight or even over a few years. In fact, obesity is deeply rooted in complex societal, cultural, psychological, and genetic trends (1–4). It has been growing stealthily for decades as a side effect of progress and prosperity. Today, 120 million Americans (64.5% of the adult population) are overweight (5). These people are our family members, our neighbors, and our coworkers.

Although being overweight or obese is still only a cosmetic issue for some people, most people who are above their recommended weight have at least an inkling that they are courting an increased array of health risks (6). Nutritionists and other health care professionals, however, know for a fact that healthy eating habits together with other healthful lifestyle behaviors have the potential to reduce the risk of chronic disease.

The food and beverage industry recognizes that it can play a powerful role in changing the eating behavior of Americans. Not only do food products influence consumer behavior and eating trends, but consumers consistently demonstrate fierce loyalty to their favorite food and beverage brands. In other words, the industry has consumers’ attention. The question is, what are they doing with it?

INDUSTRY-WIDE ACTION
Food and beverage companies are accustomed to meeting a constant demand for new and improved products that combine taste, convenience, and nutrition. Historically, the industry has been quick to respond when called on to support public health goals. For example, in tandem with Healthy People 2000 (7), in 1991, the industry was called on to introduce 5000 new reduced-fat food products by the year 2000. In fact, food and beverage manufacturers met this goal by 1995.

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Today, industry is once again stepping up to the challenge, both as individual companies and as a group. One of the most visible actions that brings industry’s collective commitment to life is the formation of the American Council for Fitness and Nutrition (ACFN).

Formed in January 2003, ACFN is a broad coalition of food and beverage companies, trade associations, and nutrition advocates that are working together toward comprehensive and achievable solutions to the nation’s obesity epidemic (8). ACFN has >90 members, including The American Dietetic Association and the American Association of Diabetes Educators, and is guided by an advisory board of experts in the fields of nutrition, physical activity, and behavior change.

ACFN brings a consensus perspective on moderation and behavior modification to the obesity debate, balancing the voice of those who look for quicker perhaps less sustainable solutions. Accordingly, ACFN’s message is one that all nutrition and other health professionals can embrace: energy balance.

Americans seem to have lost track of energy balance as a fundamental axiom of good health. To counter this trend, ACFN works with health professionals, educators, governments, policy makers, and consumers to develop lasting approaches to restore balance to the American diet. The council encourages its members to provide parents, teachers, and children with information and resources to assist them in making healthy lifestyle adjustments regarding physical activity and nutrition. ACFN also advocates for increased physical activity for every American, with an emphasis on giving students the opportunity to engage in 30 min of physical activity each day.

Finally, ACFN has also focused a portion of its most recent efforts on underserved populations, specifically the African American and Hispanic American communities that are disproportionately affected by the obesity epidemic. To this end, ACFN collaborated with local leaders to educate and encourage as many citizens as possible regarding the importance of healthy lifestyle choices. For example, in Baltimore, ACFN collaborated with the Boys and Girls Club of Central Maryland and the Baltimore International College School of Culinary Arts on a community-based summer program for urban minority youth. The 8-wk initiative was a turnkey for future programs, and it featured weekly interactive cooking segments, basic nutrition instruction, an interactive educational component, and fitness activities to help empower children to incorporate healthy eating and fun physical activity into their daily lives.

In June 2004, ACFN partnered with the National Supermarkets Association to host an educational festival for Hispanic families in Corona, NY. Nearly 400 families participated in the event, which included healthy lifestyle information and practical nutrition tips, physical activity for the whole family, and a healthful and culturally relevant food demonstration by noted “Nuevo Latino” cuisine chef Rafael Palomino.

Although ACFN is illustrative of industry-wide collaboration, there are several other significant examples of food and beverage industry commitment. For example, the industry has pledged to communicate clearly in labeling, packaging, and advertising to enable consumers to make informed choices that best meet their lifestyle needs and physical activity levels (9). In addition, the industry supports the proposals of the US Food and Drug Administration (FDA) to require labeling of trans fat and to allow more health claims. It also has petitioned the FDA to set regulatory standards for the full range of carbohydrate nutrient-content claims.

As a group, food and beverage companies are also conducting research that looks at the way consumers use the food label for nutrition information. This study will provide the FDA with valuable assistance in developing labels to better communicate calorie and serving size information. Furthermore, in line with the US Dietary Guidelines and the Food Guide Pyramid, the food and beverage industry is urging that Americans be advised to eat less, be more active, or do both to maintain a healthy weight.

The food industry is also actively engaged in the national obesity discussion, advocating policies that emphasize energy balance by bringing awareness to calories consumed and expended. For example, in Congress, the industry supported the “Improved Nutrition and Physical Activity Act,” more commonly known as IMPACT legislation, as well as the Carol M. White Physical Education Program Grants. Both provide funding for community programs that help people make good choices about food and physical activity.

**COMPANY-SPECIFIC ACTION**

Specific food and beverage companies are also taking individual action to address consumer health and wellness through products, policies, and programs. A look at supermarket shelves reveals evidence of what these companies are doing. 1) Some are reformulating products to reduce calories, trans fat and sugars, lower cholesterol, add vitamins, and preserve taste, aroma, and mouth feel. 2) Others are offering smaller portion sizes; in fact, several companies are introducing 8-oz (240 mL) beverages and single-serving snack packages. 3) Some companies are communicating clearly in labeling, packaging, and advertising to enable consumers to make informed choices that best meet their lifestyle needs and physical activity levels. Two companies have introduced extensive labeling initiatives flagging products that are smart choices based on generally accepted criteria for fat, cholesterol, sodium, and added sugar. 4) Companies are promoting nutrition education and physical activity programs, with an emphasis on schools and local communities. One company alone has committed $5 million in funding for such programs.

**NOW AND AGAIN**

In making more better-for-you foods that can easily be incorporated into daily diets, companies are seeding an environment in which consumers can learn a “new normal”: a new way to eat for a lifetime, not just a few weeks. Additionally, by supporting programs that promote nutrition education, physical activity, and physical education, companies are helping consumers continue to make smart lifestyle choices.

More and more consumers are realizing two truths that nutrition professionals have always known. First, that food and health are intimate partners. Second, that energy balance, through food and activity, is a fundamental principle of good health. As stakeholders in the health of Americans, all of us (government, advocacy groups, researchers, teachers, and industry) must collaborate to keep these messages alive and keep our family, friends, and neighbors well.

SF is chair of the American Council for Fitness and Nutrition, which is supported by the food and beverage industry.
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When science met the consumer: the role of industry1–4

Donald Short

ABSTRACT
The Coca-Cola Company is committed to understanding consumers and providing solutions to help them live active and healthy lifestyles. The Coca-Cola Company has developed a wide range of innovative beverages that can be incorporated into a healthy diet. Product offerings include beverages with fewer calories, smaller package sizes, and expanded beverage fortification options. In addition, the company has policies in place that responsibly address advertising to children and guidelines for selling beverages to schools. Nutrition education messages continue to be delivered through the Internet and on product packaging while physical activity initiatives are promoted in schools and communities. The most recent initiative has been the creation of The Beverage Institute for Health & Wellness. This institute comprises nutritionists, food scientists, physicians, and communicators and has been established with the holistic goal of providing people all over the world with a wide range of healthy beverages options. The institute supports scientific research and consumer education that focuses on the role of beverages in a healthy lifestyle. Current projects of the institute include the role of hydration in health and performance, alternative beverage sweeteners, capturing the natural goodness of fruits and vegetables for beverage use, and appropriate beverage fortification. Am J Clin Nutr 2005;82(suppl):256S–8S.

KEY WORDS The Beverage Institute for Health & Wellness, consumers, food industry, obesity, healthy lifestyle choices, marketing

INTRODUCTION
Consumers often struggle with making the right diet choices for themselves and their families while balancing an occasional indulgence in their favorite foods. The food and beverage industry can play a positive role in helping people make smart choices by providing broader selections of products, smaller packaging sizes, and product nutrition information on packaging. By providing information on the wider subject of healthy lifestyle choices, to include food and beverage choices, and by promoting physical activity, the food and beverage industry can help people make healthier choices. As a marketing company, The Coca-Cola Company spends a great deal of time understanding consumer needs on such issues. Based on these insights, The Coca-Cola Company is addressing these consumer needs through four primary avenues: 1) product innovation to provide more beverage choices and variety; 2) programs and policies, particularly in the schools; 3) physical activity, nutrition, and lifestyle education programs; and 4) a science-based Beverage Institute for Health & Wellness.

PRODUCT INNOVATION
One focus of Coca-Cola’s product innovation efforts is to provide lower-calorie products, smaller package sizes, and beverage fortification. The Company’s global beverage portfolio, of 400 brands, comprises soft drinks, diet soft drinks, juices, juice drinks, sports beverages, waters, teas, coffees, milk-based drinks, and fortified beverages (1).

In the lower-calorie beverage category, the company has built on its success with Diet Coke, the third largest-selling soft drink in the world (2). The Company has introduced several new flavors of this well-known diet soft drink, as well as fruit juice-based drinks such as Minute Maid Light Lemonade, in bottles and cans, with only 5 cal per 8 fl ounce serving. Furthermore, the Company has made regular soft drinks available in 8 oz cans, providing a way for consumers to enjoy a soda but in a more limited portion size.

The company also has been involved in a substantial amount of innovation in beverage fortification. Orange juice, in particular, has proven to be an excellent vehicle for delivering fortification because it is so frequently consumed. The company introduced Minute Maid Premium orange juice fortified with calcium in 1986. More recently, using the National Health and Nutrition Examination Survey (NHANES III, 1988–1994) and Continuing Survey of Food Intakes by Individuals (CSFII 1994–1996, 1998), the Company worked with ENVIRON Health Sciences Institute to create a vitamin D database to estimate mean intakes of the entire United States population (3). A significant gap in the intake of vitamin D compared with recommended levels was identified. Because vitamin D is a fat-soluble vitamin, it was thought that only beverages containing fat could be fortified with vitamin D. Because vitamin D plays an important role in increasing calcium absorption, a study was undertaken to assess whether or not vitamin D would be bioavailable when added to calcium-fortified orange juice (4). Findings indicated that vitamin D was bioavailable, and the company submitted a food additive petition to the Food and Drug Administration (FDA) to change regulations to allow for the addition of vitamin D to calcium-fortified juices and juice drinks (5). In 2003, the

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FDA approved the food additive petition, and Minute Maid Premium orange juice fortified with calcium plus vitamin D was launched. Continuing the effort to explore ways to enhance calcium absorption, the company participated in a clinical trial designed to investigate the possible benefit of inulin-type fructans on increasing calcium absorption from fortified orange juice in pubertal adolescents. Results indicated that inulin-type fructans significantly increased calcium absorption and enhanced bone mineralization during pubertal growth (6).

The Coca-Cola Company distributes beverages in almost 200 countries that include both developed and developing markets. Recognizing that micronutrient malnutrition is an important health concern in many developing countries, the company undertook a study in Botswana to determine whether a micronutrient beverage containing 12 essential vitamins and minerals could improve nutrition status in children who were at risk for micronutrient deficiency. Findings demonstrated significant improvements in iron, zinc, folate, and riboflavin status (7). A follow-up study in Peru demonstrated that both iron and zinc in the micronutrient beverage were also bioavailable when given with a meal (8). Since efficacy and bioavailability had been confirmed, the company introduced Vitingo in South Africa in 2002 to provide consumers with an affordable beverage to address common micronutrient deficiencies.

In 2004, the company introduced Minute Maid Premium Heart Wise orange juice with plant sterols, which, through a clinical study, was proven to lower LDL cholesterol. Study participants with normal to borderline high cholesterol levels significantly lowered their LDL cholesterol 12.4% by drinking two 8 fl oz (240 mL) servings of Minute Maid Premium Heart Wise orange juice per day with meals for 8 wk (9). To educate consumers regarding the benefits of plant phytosterols, Minute Maid Heart Wise packaging used the FDA-approved health claim that “foods containing at least 0.4 g per serving of plant sterols, consumed twice a day with meals for a daily total intake of at least 0.8 g, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease” (10). A serving of Minute Maid Heart Wise contains 1 g of plant sterols per 8 fl oz (240 mL) serving.

PROGRAMS AND POLICIES

The Coca-Cola Company and its bottlers collaborated with leaders from major educational organizations to develop a set of guidelines for selling beverages to schools. These guidelines cover numerous aspects of school contracts, such as appropriate product mix availability in vending machines and appropriate branding in the school environment. The company has recommended that carbonated soft drinks not be available to elementary school children during the school day and has made timers available so that schools can control the availability of beverages to students at appropriate times. In middle and high schools when carbonated soft drinks are made available, a full array of other products also must be available, such as juices, water, and sports beverages. Ultimately, the decision as to which Coca-Cola beverages to offer and when is determined by local school boards, teachers, and parents acting within Federal guidelines. The Company respects the classroom as a commercial-free zone. Company logos and other marketing graphics are not permitted on textbooks, curriculum materials, or book covers. Venues that are appropriate for product logos include scoreboards, menu boards, coolers, student publications, and materials to promote educational activities, health wellness and nutrition education, extracurricular activities, physical activity, and athletic events. These are but a few examples of the company’s interest and intent to work in concert with the education community, at both a national and local level.

The company continues to respect the authority of parents and caregivers to make choices for their children. Recently, The Coca-Cola Company reaffirmed its 50-y-old policy regarding marketing carbonated soft drinks directly to children under 12. For example, the company does not place advertising for any brands during Saturday morning or after-school television, in print media primarily directed to younger children, or in venues frequented primarily by unsupervised children.

PHYSICAL ACTIVITY AND NUTRITION EDUCATION

The Coca-Cola Company and almost 80 Coca-Cola bottlers nationwide also support school and community physical activity programs in their local regions. The company sponsors national programs such as Step With It! in middle schools. Step With It! provides stepometers to students in middle schools to encourage them to get more active as part of an overall healthy, active lifestyle. By the end of 2004, this interactive program reached one million students and educators. In 2005, the Step With It! program will be expanded to include both a physical activity and a nutrition-education component.

The company also has implemented a highly successful community-based youth soccer program called Copa Coca-Cola for boys and girls throughout the Americas and Europe. In 2004, ~10 000 teams and >600 000 student athletes participated in the youth soccer program. Copa Coca-Cola originated in Mexico, and the program helps to promote the need for physical activity outside the school grounds. The company also has supported the Tiger Woods Foundation in a program that brings golf clinics to inner city youths who otherwise might not be exposed to golf. Recently, the company made a multiyear commitment to the Boys & Girls Clubs of America to sponsor a new program called Triple Play in conjunction with Kraft Foods and the US Department of Health and Human Services, which includes physical activity, nutrition education, and life skills for youths.

The Coca-Cola Company continues to participate in many clinical studies in the area of nutrition and physical activity. The company helped sponsor the Beat Osteoporosis Nourish and Exercise Skeleton (BONES) nutrition and exercise intervention program conducted by Tufts University. Minute Maid calcium-fortified juices were provided to 1470 children aged 6–9 y who participated in after-school programs for a period of 25 wk. Preliminary results indicated that fortified juices contributed significantly to the increased calcium of 1191 mg/wk (11).

The company also has a longstanding interest in the ability of beverages to provide hydration, during both physical activity and normal activities of daily living. Research supported by the company helped demonstrate that carbonated soft drinks with and without caffeine can contribute to the hydration needs of healthy adult males (12). These findings were cited in the Institute of Medicine’s Dietary Reference Intake recommendations for water and helped support the final conclusion that caffeinated beverages appear to contribute to the daily total water intake, similar to that contributed by noncaffeinated beverages (13).
The company continues to use the Internet as a great platform for physical and nutrition education. The company has long supported www.kidetic.com, a site designed for kids to promote physical fitness and activity. For several years, the company sponsored www.MayoClinic.com and has included health tips from the site on millions of cartons of Minute Maid Premium orange juice products. This collaboration has been an excellent means for delivering health and nutrition messages to consumers in the comfort of their own kitchen.

THE BEVERAGE INSTITUTE FOR HEALTH & WELLNESS

One of the company’s most recent initiatives was the creation of The Beverage Institute for Health & Wellness. The goal of the institute is to help people all over the world by providing a wide range of healthy beverages options. This organization, comprising nutritionists, food scientists, physicians, and health communicators, is funded and operated by The Coca-Cola Company. The institute has a global focus, revolving around health and nutrition. The institute continues to identify insufficient nutrient intakes in various populations, in different parts of the world, and continues to conduct clinical work to study how beverages may play a role in filling those nutrient intake gaps. The results of the clinical work, typically conducted at independent universities and institutions, helps to support the future development and the ultimate launch of new beverages.

The institute is served by an international advisory council made up of global thought leaders with expertise in several areas that include nutrition, medicine, exercise physiology, consumer research, culinary arts, and retailing. The advisors help to set research agendas and guide the scientific integrity of the work. Current primary areas of interest for the institute include expanding the universe of sweetener knowledge, increasing the understanding of the natural goodness of phytochemicals in fruits and vegetables that may have an application in beverages, advocating hydration as a key component of a healthy diet, expanding appropriate beverage fortification innovation, and demonstrating beverage efficacy through clinical trials.

The institute seeks to educate and inform consumers, health professionals, and media on topics relevant to beverages. In an effort to help increase the awareness of new research on vitamin D and health, in 2003, the company helped sponsored a National Institute of Health conference on “Vitamin D and Health in the 21st Century”. The Beverage Institute for Health & Wellness contributed funds to help publish the conference proceedings (14). In 2004, the institute helped sponsor a symposium on vitamin D insufficiency as a significant risk factor in chronic diseases during Experimental Biology meetings in Washington DC. Again, the Institute helped publish the vitamin D symposium proceedings (15).

CONCLUSION

The Coca-Cola Company is taking a proactive role by creating a new frontier of products by launching innovative new beverage choices, introducing new programs to help consumers navigate their way through the health and diet landscape, and establishing the science-based Beverage Institute for Health & Wellness. Research findings and health-promotion activities, conducted by The Beverage Institute for Health & Wellness, are now available at www.thebeverageinstitute.org. We believe these efforts are steps in the right direction toward helping consumers lead healthier lives.

I thank Carolyn Moore and Donna Shields for assistance with manuscript preparation. DS is the Vice President of The Coca-Cola Company and Director of The Beverage Institute for Health and Wellness.

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Solutions to obesity: perspectives from the food industry

Patricia Verduin, Sanjiv Agarwal, and Susan Waltman

ABSTRACT
Obesity has become an epidemic and an important public health concern. Because the problem is multidimensional, the solution will require an interdisciplinary approach involving the cooperation of the food industry with other stakeholders, such as the government, academia, and health care providers. The consumer is an important player in the solution to obesity because the consumer can make healthy lifestyle choices at the individual level. The food industry is committed to providing the consumer with healthy food options and reliable nutrition information.

KEY WORDS Food industry, obesity, consumer choices, consumer attitude, dietary decisions, food labeling, healthy products

INTRODUCTION
Obesity has reached epidemic proportions in United States. Currently, more than 65% of Americans are overweight or obese (1). Obesity is correlated with several medical conditions, including type 2 diabetes, heart disease, high blood pressure, stroke, and certain types of cancer. Obesity and its related diseases are responsible for ≈400 000 deaths per year in the United States, paralleling the number of preventable deaths caused by smoking (2). Overweight in pediatric age groups has nearly tripled in the past 30 y (3). Today, an estimated 16.1% of adolescents (12–19 y of age) in the United States are overweight (body mass index ≥125th percentile for age) (4). Studies indicate that 50–77% of these adolescents will become obese adults; 80% of those with one obese parent will do so (5–8). Data show significantly lower quality of life scores for obese children compared with children of normal weight (9). They also show increased risk of obesity-related comorbidities, including degenerative joint disease (10, 11) and type 2 diabetes (5, 12–14).

Obesity is a multifaceted health issue that involves biological, behavioral, and environmental sources. Energy imbalance sits at the core of the obesity problem, because weight gain results from consuming more calories than one expends. In America, a changing environment has increased food choices and changed eating habits. Many Americans are sedentary: more than one-half of US adults do not meet recommended levels of moderate physical activity, and one-fourth engage in no leisure time physical activity (15). Technological inventions have created many time- and labor-saving products. As a result, we have reduced the overall energy expenditure in our daily lives. Critical approaches to weight reduction involve behavior change related to diet and exercise. Stakeholders, including the food industry, government, academia, and health care providers, can work together to influence the consumer to make healthy lifestyle choices. ConAgra recognizes the influence the food industry has on consumer choices.

ROLE OF THE CONSUMER
Consumers are the most important player in the solution to the obesity epidemic because they make individualized choices about food and lifestyle. As stated by Philipson in this symposium (16), if the consumer can be influenced with comprehensive nutrition information and a variety of healthy food choices, perhaps individuals can begin to address weight gain on a personal level. In addition to cultural and psychological influences, four motivators have been identified that affect consumer decisions: taste, quality, convenience, and price. Although consumers indicate that healthy eating and good nutrition are increasingly important to them, sales and surveys show they are more concerned with taste, convenience, and price (17, 18). There exists a gap between consumer attitude and behavior that the food industry must consider. Moreover, consumers are confused with the conflicting messages regarding fat, carbohydrates, protein, and calories. Consumers need clarity and reliable nutrition information to make responsible dietary decisions.

ROLE OF THE FOOD INDUSTRY
The food industry should help the consumer make healthy food choices. Food manufacturers are sensitive to consumer tastes and expectations. Industry should be committed to giving clear, consistent, and honest product claims, as well as working with retailers and restaurants to offer consumers relevant information about the products they purchase. Industry should promote nutrition education at all levels, from public schools to medical schools, and enhance nutrition awareness at the consumer level. Industry can create new products that meet individual nutritional needs, reformulate existing products to be healthier, and provide controlled portion sizes. In doing so, industry faces the challenge of satisfying consumer expectations for taste, quality, and price. The hope is that industry can develop healthier

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products by partnering with science-based communities and the government.

INDUSTRY COMMITMENT TO HEALTHY LIVING

The food industry recognizes the challenges a consumer faces when making food choices. ConAgra has developed new food products and improved old favorites to offer consumers a large range of healthy food options. The variety within this range of healthy products provides consumers with the flexibility to satisfy different dietary needs and different lifestyles.

The Healthy Choice product line began in 1988 and now has over 200 healthy products available. Healthy Choice provides consumers with low-calorie, nutritious meals, deli meats, breads, soups, and desserts. Healthy Choice “Flavor Adventures” are frozen meals with exciting seasonings and ingredients that enhance taste without compromising healthful benefits and nutrients.

Egg Beaters is another popular, tried-and-true item within the good-for-you product range. Egg Beaters is a real egg product that features no fat, no cholesterol, and half the calories of regular eggs, in addition to the ease of reusable packaging, which gives consumers another quick and healthy food option.

The PAM line of no-stick cooking sprays now includes fat-free original, fat-free olive oil, and fat-free “for grilling” varieties, which promote healthier and hassle-free options for cooking and grilling.

Fleischmann’s margarine products, targeted at health-conscious consumers, offers Light, Unsalted, and No Trans fat cholesterol-free alternatives to butter.

Hunt’s Tomatoes and their foodservice counterpart Angela Mia have teamed with the American Dietetic Association to promote the health benefits of tomatoes, which are rich in lycopene. Hunt’s also offers organic tomatoes.

The Lightlife brand caters to vegetarian needs with meatless, soy-based products.

ConAgra now features a new line called LifeChoice for consumers who want meals low in carbohydrates.

Ultragrain, a creation of the ConAgra Ingredients group, combines the nutritional value of whole grains with the taste and texture of refined grains to enrich the diets of consumers who prefer refined grains.

ConAgra promotes healthy living in many other capacities, including a Healthy Choice website at www.healthychoice.com and a monthly email newsletter called “To Your Health” that features coupons, recipes, and nutritional tips.

CONAGRA’S COMMITMENT TO THE COMMUNITY

In addition to providing consumers with a multitude of healthy food options, ConAgra remains committed to helping the environment and communities in need. ConAgra’s “Feeding Children Better” foundation has funded over 160 Kids Cafes, providing logistics assistance and food donations for millions of “food insecure” American children.

ConAgra has joined with the American Dietetic Association to promote Home Food Safety and to decrease the prevalence of food-borne diseases.

COOPERATION WITH THE GOVERNMENT

ConAgra stresses the importance of a close harmony between the food industry and other important stakeholders within the obesity epidemic, such as the government, academia, and health care providers. The government can help educate consumers to make healthier choices through food labeling, physical activity endorsement, and the support of community-based programs.

The food industry should cooperate with the government to guarantee the production and availability of low-calorie, nutritious foods. This can be achieved by providing incentives to industry and also stimulating media participation in the prevention of obesity. The government should develop and support new food technology and improve communication across responsible parties and consumers.

COOPERATION WITH ACADEMIA AND HEALTH CARE PROFESSIONALS

ConAgra relies on the expertise of scientists and health care professionals within the medical field. Scientists have a unique insight to provide scientific data influencing the decisions of industry and consumers. Research scientists can work together with the food industry to generate nutritious foods and to promote reliable, science-based nutrition information. Academic institutions also play a key role in educating health care professionals, who will educate their patients and our consumers. The medical community is uniquely positioned and trusted by consumers, because consumers look for nutritional advice from knowledgeable and trustworthy professionals.

Health care providers can encourage consumers to make individual lifestyle changes that will improve their health. The health care professional, in assuming such a role, can transform their interaction on this topic from clinical resolution to thoughtful prevention, thus helping consumers develop and reinforce strategies for eating and physical activity that reduce overall individual health care risk.

CONCLUSION

To help fight the battle against obesity, the food industry must team up with the government, academia, and the medical community to help inform consumers, strengthen nutrition education, and develop healthier product choices. ConAgra aims to work in concert with these groups to continue providing consumers with a range of healthy food options. Giving consumers the best nutrition information and the best nutritional food options may empower them to make individualized lifestyle changes that will help overcome energy imbalance and, in the long term, may help curb our struggle with obesity.

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Government perspective: food labeling1–3

Tomas Philipson

ABSTRACT

The Food and Drug Administration acknowledges the severity of the obesity epidemic. The Food and Drug Administration recognizes the importance of food labeling as a vehicle for dietary messages and, thus, enforces stringent guidelines to maintain the integrity of the food label. As food labels await another upgrade to make them more effective and easier to understand, the Food and Drug Administration considers what information will be most useful for consumers to make healthy choices. The causal relationship between food labels and subsequent diet choice is not well understood; more research in this area is needed. The Commissioner of the Food and Drug Administration has recently appointed an Obesity Working Group to develop proposals on pertinent topics of obesity, including the role of food labeling as a dietary guide. Am J Clin Nutr 2005; 82(suppl):262S–4S.

KEY WORDS Food labels, nutrition labels, Food and Drug Administration, dietary guide, Obesity Working Group, calories count, health claims, consumer advice

INTRODUCTION

The Food and Drug Administration (FDA) recognizes the obesity epidemic as an urgent public health issue, and we are committed to the search for solutions. The experience of the Food and Drug Administration regarding the utility of food labeling as a dietary guide is an important component of the blueprint for a healthier United States. The FDA’s attempt to use the food label for dietary advocacy goes back to 1989. At that time, the food label had not been updated for almost 20 y, its format was anybody’s choice, and labeled health claims were both exuberant and undocumented. The Surgeon General’s Report on Nutrition and Health in 1988 instigated the label change. The FDA decided to develop a new food label that would be uniform, science based, and rigorously truthful. The updated food label would inaugurate a new era in food labeling by telling shoppers the main ingredients in every manufactured food item and how much of that food was good for their health. The Nutrition Facts Panel is now a familiar component of the food label.

After Congress endorsed this effort in the Nutrition Labeling and Education Act of 1990 (1), the FDA launched one of the most extensive undertakings in its history. The agency held nationwide hearings to gather facts and expert views, considered scores of scientific documents and studies, drafted nearly 900 pages of proposed regulations, analyzed 40 000 comments submitted by leaders in food science and industry, and, in 1993, produced a food label that was designed to help the public chose a low-fat, high-fiber daily diet of 2000 or 2500 calories. The climax of the effort was a coast-to-coast, multi-lingual educational campaign launched in March 1994 and delivered the FDA’s message into virtually every American household. It was projected that the new label would save the nation up to $26 billion in health care costs over 20 y and prevent many of the 300 000 deaths per year that were then associated with diet. The results have not lived up to the early expectations entirely, but they have not been uniformly bleak either.

Surveys report 60–80% of food shoppers had read food labels before buying a new food item, and 30–40% said the label had influenced their choice. The surveys also revealed frequent misunderstanding of the meaning of the daily/value column that shows how each nutrient fits into a healthy diet (2–6). There was also considerable confusion caused by the frequently conflicting dietary advice by nutritionists, fad diet advocates, and studies reported by the media. This bewilderment has generated distrust of all dietary recommendations and a corresponding desire for nutrition information that is clear, authoritative, and easy to understand.

UPGRADING THE FOOD LABEL

The FDA decided to undertake an effort to make the food label a more effective vehicle for dietary messages. Our first step was a proposal, issued in December 2002, to relax the enforcement of a provision of the Nutrition Labeling and Education Act requiring that all health claims on food labels be supported by a “significant scientific agreement.” This standard has been so difficult to achieve that the FDA has approved only 12 unqualified cause-and-effect health claims in the past 10 y, such as the linkage between calcium and osteoporosis, fat and cancer, and folic acid and neural tube defects. Two more health claims were added under a new law that allows such labeling based on an “authoritative statement” from the National Academy of Sciences or a scientific body of the federal government. The legislated restrictions have prevented manufacturers from labeling claims based on emerging, and therefore incomplete, scientific evidence, such as the well-documented findings that omega-3 fatty acids in certain kinds of fish can help reduce the risk of coronary heart disease (7).

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FOOD LABELING

The Obesity Working Group topics

- An effective public health message about the need to balance food intake with expended energy
- A consumer education program with the same aim
- Better food labeling
- Research on consumer behavior in regards to food consumption
- Enhancement of the development of effective weight-loss and weight-control drugs and devices

Source: Reference 9.

The Obesity Working Group found little evidence that indicates the effects the food label may have on obesity (10). There seems to be a need for well-controlled studies that examine the effects of food labeling on diets. Current labeling, on the product level, does not facilitate learning about diets, which come from a combination of products. Therefore, the Obesity Working Group considered restaurant labeling as a partial remedy to this problem by labeling entire meals rather than single products. We also considered better labeling of diets and weight-loss products. Consumers spend billions of dollars per year on these products, although many studies show that diets fail for 95% of those who try. If diets are “treatments” for weight reduction, then the “intent-to-treat effects” of these treatments are very poor, partly attributable to the difficulties with compliance or adherence to the treatment prescribed. The following question arises: if consumers were better informed of the intent-to-treat effects of these products, would the demand remain as high?

The Obesity Working Group, in conjunction with advice from its stakeholders, questioned how the current Nutrition Facts Panel on packaged food could better persuade or influence consumers to maintain their energy balance. The suggestions confirmed that food labeling should be an effective vehicle for raising the public’s consciousness of the importance of calories when making dietary decisions. For example, the FDA was urged to encourage manufacturers to indicate the total caloric content on the food label of an entire food package if it is likely to be consumed in one sitting. The FDA was also asked to urge food firms to print the caloric count in bold digits on the face of each food package, to encourage labeling statements suggesting alternative food choices with fewer calories, and to develop labeling statement that would inform the consumer how much exercise would be necessary to burn the calories in the food package. A label was also proposed that would list the health consequences of eating too much, something similar to the Surgeon General’s warning on packs of cigarettes. These ideas require substantial research on consumer responses to labeling.

CONCLUSION

The FDA invites any and all present behavioral scientists who are interested in these issues to assist us. We need better-controlled studies of the effects of labeling on the demand for different foods. There are many studies on this topic, but most of them focus on correlations of labeling use and diet choice. Such correlations may be spurious when unobserved factors drive both the demand for label information and diets. This would be the case when health-conscious people, who already have healthy diets without the label, are observed to have healthy diets when using the label. We also need better-controlled experimental designs to assess predemand and postdemand behavior surrounding a change in labeling, randomly assigned to different parts of the consumer population. Such studies would be extremely helpful; the FDA hopes to work with manufacturers to conduct them in the future.

The use of food labeling for dietary advocacy is an endeavor the FDA intends to advance very intensively, imaginatively, and as efficiently as possible, but also with great care. Without solid scientific conclusions bearing on the issues of food labeling and the obesity epidemic, the proposals would not be in the interest of public health or in the tradition of a science-based agency such as the FDA.

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The economics of obesity: dietary energy density and energy cost1–4

Adam Drewnowski and Nicole Darmon

ABSTRACT

Highest rates of obesity and diabetes in the United States are found among the lower-income groups. The observed links between obesity and socioeconomic position may be related to dietary energy density and energy cost. Refined grains, added sugars, and added fats are among the lowest-cost sources of dietary energy. They are inexpensive, good tasting, and convenient. In contrast, the more nutrient-dense lean meats, fish, fresh vegetables, and fruit generally cost more. An inverse relationship between energy density of foods (kilojoules per gram) and their energy cost (dollars per megajoule) means that the more energy-dense diets are associated with lower daily food consumption costs and may be an effective way to save money. However, economic decisions affecting food choice may have physiologic consequences. Laboratory studies suggest that energy-dense foods and energy-dense diets have a lower satiating power and may result in passive overeating and therefore weight gain. Epidemiologic analyses suggest that the low-cost energy-dense diets also tend to be nutrient poor. If the rise in obesity rates is related to the growing price disparity between healthy and unhealthy foods, then the current strategies for obesity prevention may need to be revised. Encouraging low-income families to consume healthier but more costly foods to prevent future disease can be construed as an elitist approach to public health. Limiting access to inexpensive foods through taxes on frowned upon fats and sweets is a regressive measure. The broader problem may lie with growing disparities in incomes and wealth, declining value of the minimum wage, food imports, tariffs, and trade. Evidence is emerging that obesity in America is a largely economic issue. Am J Clin Nutr 2005; 82(suppl):265S–73S.

KEY WORDS Obesity, poverty, energy density, energy intake, food prices, diet cost, economics

INTRODUCTION

Energy-dense foods and energy-dense diets have been blamed for the global obesity epidemic (1–5). In a number of studies, fast foods (6–9), snacks, sweets, and desserts (10, 11), sweetened soft drinks (12–14), and large portion sizes (15, 16) have all been linked to greater obesity risk.

Studies on obesity and the food environment have focused on the interaction between human physiology and the changing nature of the food supply. At different times, corn sweeteners (12), sucrose (17), protein (18), fat (19), and starch (20) were all said to promote overweight through a variety of metabolic mechanisms. Physiologic systems regulating food intake were said to be at fault. Whereas some researchers suggested that humans fail to compensate for calories in energy-dilute beverages (21), others blamed our weak innate ability to recognize calories in energy-dense foods (8). Whereas some implicated the consumption of sucrose (17), others reported that high-fructose corn syrup was responsible for high obesity rates (12). Still others suggested that the body’s natural ability to count calories was impaired by noncaloric sweeteners (22). Where the food was eaten was another contributing factor. Whereas some reports identified away from home foods and restaurant meals as a potential cause of obesity (6, 7, 23, 24), others pointed to the key role of between-meal snacks (10) and growing portions of foods consumed at home (25).

In other words, published scientific research suggests that obesity is caused by the following: excessive consumption of protein, starch, sugar, and fat; by caloric and noncaloric sweeteners; by meals and by snacks; by beverages and by solid foods; by eating in fast-food and in full-service restaurants, as well as by eating at home. Only vegetables and fruit have not been linked to higher obesity rates, as yet (26, 27).

Modifying the food environment has risen to the top of public policy agenda (28, 29). Many such efforts have focused on removing the offending foods from the consumers’ reach. Fear of the “toxic” food environment has led to proposed taxes on fats and sweets, to both discourage consumption and promote alternative healthier diets (30). Policy approaches to improving nutrition at schools have called for limiting access to vending machines containing beverages and snacks (3, 14, 31) and regulating the sale of competitive foods. Agricultural policy options include the provision of economic incentives for the production of healthier foods (31, 32) and the removal of existing subsidies. In addition, various sectors of the food, grocery, and restaurant business have found themselves exposed to lawsuits for their alleged role in causing obesity among their customers (33–35). Although most lawsuits were dismissed, future plaintiffs may

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benefit from novel legal theories linking the food environment to greater obesity risk (36).

Although the dramatic rise in obesity rates can only be explained by environmental factors, there has been little emphasis on the obese persons’ economic environment (24). In particular, there has been little research on diet quality and the economics of food choice (37–39). Existing studies suggest that the high cost of healthier diets may contribute to the obesity epidemic, especially among the lower-income groups (40, 41). Examining the links between dietary energy density, food prices, and diet costs is the chief focus of this report.

HOW DO PEOPLE MAKE FOOD CHOICES?

Identifying factors responsible for food selection should precede any effort at dietary change. Food choices are made on the basis of taste, cost, and convenience, and, to a lesser extent, health and variety (42). Taste refers to the sensory appeal of foods, such as palatability, aroma, and texture (43). The concepts of taste and energy density are intertwined, because the most energy-dense foods are usually the most palatable and vice versa (44). Energy density of foods is defined as the energy per unit weight or volume (kcal/100 g or megajoules per kilogram). Cost refers to the purchase cost per unit of energy (Euros/1000 kcal or dollars per megajoule) or the purchase cost of a daily diet (Euros of dollars per day). Convenience refers to the time spent on buying, preparing, and cooking food. Variety refers to the innate drive to secure a varied diet, whereas health refers to concerns with nutrition, chronic disease, and body weight. A model representing these factors is shown in Figure 1.

The literature on health promotion has emphasized the psychosocial aspects of food selection (45–47). The unspoken premise has been that adherence to healthy diets is essentially a matter of awareness, motivation, and making the right food choices. Largely missing from the literature has been any mention of limited economic resources and high diet costs (48). The average American spends less than $8.00/d on food and beverages, with low-income families spending as little as $25 per person per week (49). Although not all food purchases are price driven, each adult needs to obtain an energy ration of 2000–2500 kcal (8.32–10.4 MJ) each day at an affordable cost.

Researchers at the US Department of Agriculture (USDA) have pointed out that the American diet is inconsistent with the Food Guide Pyramid (50). The consumption of fats and sweets at the Pyramid’s tip far exceeds recommendations, especially when compared with the low use of green leafy vegetables and fruit. There is a reason why refined grains, fats, and sweets have come to dominate the food supply. They are good tasting, energy dense, convenient to use, and inexpensive (49). Limited financial resources may be one reason why people are not eating more healthfully (40, 41).

In general, healthier diets cost more. Developments in agriculture and food technology have made added sugars and vegetable oils accessible globally at a remarkably low cost. The cost of producing sugar is Brazil is as low as $4/e/lb (9/e/kg) (51). The commodity cost of refined sugar (sucrose) in global markets is in the order of $9/e/lb (20/e/kg), whereas the cost of most vegetable oils is approximately $20/e/lb (44/e/kg) (38). In other words, ≈40 000 kcal (167 MJ) from added sugars and fats can be obtained at world market rates for only $2.00. Although there is little relationship between commodity cost and the retail cost of the finished food product, caloric sweeteners, grains, and added fats help to hold down the cost of the daily diet. Americans have the lowest cost food supply in the world. The typical American diet derives almost 40% of daily energy from added sugars and from added fats (50).

Diet quality, both in the US and elsewhere, is a function of social class. It is well known that older and wealthier consumers have higher quality, healthier, and more varied diets, with a higher proportion of high-quality meats, seafood, vegetables, and fruit (52–55). In contrast, lower-income households tend to select diets high in low-cost meats, inexpensive grains, added sugars, and added fats (56–59). In a recent study of low-income families, fruit and vegetable expenditures were low. Bananas were far more likely to be purchased than were the more expensive berries and other fruit (55). Food assistance recipients, taking part in USDA focus groups, were primarily concerned with obtaining sufficient calories at low cost, so that nobody would complain they were still hungry (60). Diet quality is influenced by socioeconomic position and may well be limited by financial access to nutrient-dense foods.

ENERGY-DENSE FOOD COST LESS

Teaching the poor how to satisfy protein and energy needs at the lowest cost was an early task of Wilbur Atwater (61). Historians regard that work as the beginning of significant nutrition research in the United States (62). At the time, working families spent ~50% of their income on food (62). Atwater’s calculations established that wheat flour and dried beans provided energy and protein at a lower cost than did either meat or fruit. This hierarchy of food prices has remained primarily unchanged in more than 120 y. Dry foods with a stable shelf life are still less costly (per 1000 kcal or per megajoule) than are perishable meats, fish, dairy, or fresh produce. However, only limited contemporary data exist on the relationship between energy density and energy cost (63, 64). One obstacle is the lack of an updated food price database for the United States.

The INCA study (Étude Individuelle et Nationale sur les Consommations Alimentaires) was a national study of food consumption conducted by the French government. We were able to assign mean national retail price to each of the 895 foods in the nutrient database. Prices for most foods (n = 760) were based on a marketing database obtained from the Société d’Études de la Consommation, de la Distribution, et de la Publicité (SECO-DIP); other prices were obtained from the French National
Institute of Statistics [Institut National de la Statistique et des Etudes Economiques (INSEE)] and from supermarket websites. Energy density (kcal/100 g) was calculated using food composition tables. Mean cost per edible portion of food was calculated, after adjusting for preparation and waste (65).

**Figure 2** shows a scatter plot of energy density of foods and their energy cost (in Euros/1000 kcal), separately for each food group. Energy cost is represented on a logarithmic scale. Fats and oils, sugar, refined grains, potatoes, and beans provided dietary energy at the lowest cost. At retail prices, energy cost of sugar or oil was in the order of 0.1 Euros/1000 kcal. In contrast, the cost per calorie of meats, fish and shellfish, dairy products, vegetables, and fruit was much higher. As indicated by the logarithmic scale, the differential in energy costs between the “healthy” and “unhealthy” foods was several thousand percent. The frowned-upon fats and sweets and the recommended fresh produce were, in reality, separated by an immense gap in energy costs.

**HEALTHIER DIETS COST MORE**

If healthier foods cost more, then so will healthier diets. Our hypothesis was that dietary energy density and daily diet costs would be inversely linked, after adjusting for energy intakes. We therefore examined, for the first time, the relationship between energy density and the cost of freely chosen diets in a French community study (63). The Val-de-Marne dietary survey, conducted in 1988–1989, used probability sampling and a two-stage cluster-design procedure (66, 67). Dietary intakes were estimated using a dietary history interview, based on daily intakes representative of a habitual diet over 6 mo (68). Food consumption was assessed in terms of frequencies (per week) and quantities consumed (portion sizes) in a manner similar to a food frequency questionnaire. The analyses were based on 837 adults aged ≥18 y (361 men and 476 women) and on 57 food items, after excluding drinking water, alcoholic beverages, and baby and infant formula products. The complete Val-de-Marne nutrient database has been published previously (66).

Dietary energy density (megajoules per kilogram) was obtained by dividing energy intakes by the estimated edible weight of all foods and caloric beverages (69, 70). Diet costs were estimated by attaching a price to each of the 57 food items. Mean national retail prices for year 2000 for each of the 57 items were provided by the French National Institute of Statistics (INSEE 2000). A column of prices in Euros (1€ = 1.17 US$ in June 2003) was added to the Val-de-Marne food composition database. The price of red meat was based on frozen hamburger, whereas the price of poultry was based on chicken breasts. The prices of hard cheese and soft cheese were based on the price of Emmental and Camembert cheeses. The vegetable category was represented by prices for potatoes, tomatoes, carrots and endives (all fresh), mixed vegetables, peas and beans (all canned), and dried lentils. The foods selected to represent the cost of diets were the more frequently consumed and lower-cost options, including some frozen and canned foods. The present method of estimating diet costs is based on the assumption that all foods were purchased and then prepared and consumed at home. The same exact assumption had been made by the US Department of Agriculture in developing the Thrifty Food Plan (71). Diet costs were estimated based on retail food prices, assuming that the foods were prepared and consumed at home (71).

Mean energy intakes without alcohol in the Val-de-Marne dietary survey were 9.89 MJ for men and 7.38 MJ for women. The more energy-dense diets were associated with a higher consumption of grains, fats, and sweets and negatively with the consumption of fruit and vegetables, after adjusting for energy. Dietary energy density (megajoules per kilogram) was associated with higher energy intakes ($R^2 = 0.31$, $P < 0.0001$), consistent with previous reports.

Mean estimated diet cost was 5.59 €/d for men and 4.63 €/d for women. That estimated daily diet cost of approximately 5 €/d was very close to the mean national expenditures for at-home foods, as calculated by INSEE from the National Budget Survey (4.9 € per person per day) (72). Energy density of the diet and energy cost were inversely linked (73). Women consumed more vegetables and fruit and had more energy-dilute diets; mean estimated energy cost per 10 MJ was higher for women (6.56 €/d) than for men (5.85 €/d) (73).

Participants were then split by quintiles of energy intake (megajoules per day), and the relationship between diet composition and diet costs was assessed separately for each quintile in a regression model, adjusted for sex and age. The question was whether replacing fats and sweets with more vegetables and fruit would be associated with higher diet costs. Figure 3 shows that,
depending on energy intakes, each 100 g increment in fruit and vegetables consumption was indeed associated with an increase in diet costs of 0.18–0.29 €/d.

In contrast, higher consumption of fats and sweets was associated with a net saving in diet costs (74). Figure 4 shows that, for persons in the lowest energy quintile, each 100 g of fats and sweets was associated with a 0.40 €/d reduction in daily diet costs. The relationship flattened as energy intake increased, but even for persons in the highest energy quintile, each 100 g of fats and sweets was associated with a saving of 0.13 €/d. In other

FIGURE 3. Relationship between fruit and vegetable consumption (grams per day) and diet costs (Euros per day). Regressions are for each quintile of energy intake.

FIGURE 4. Relationship between fats and sweets consumption (grams per day) and diet costs (Euros per day). Regressions are for each quintile of energy intake.
words, sweets and fats cost less, whereas energy-dilute diets high in vegetables and fruit cost more (74).

ESTABLISHING CAUSAL PATHWAYS

The present hypothesis is that reducing diet costs will lead to a lower-quality diet. The observed inverse association between diet quality and diet costs was based on a cross-sectional community study of French adults (73, 74). The causality hypothesis was tested using linear programming models, as applied to those freely chosen diets.

Linear programming models optimize a given function, subject to a variety of constraints. They have been used to design least-cost nutritious diets that were minimally acceptable to the consumer (75–77). Our goal was to explore the impact of economic constraints on the nutritional quality of the diet while keeping palatability high. Nutritional constraints were not included in the model whose objective was to minimize deviations from the usual French diet while progressively decreasing diet cost. The question was what kind of a diet can be obtained at low cost if economic resources are limited and the consumer is unwilling to adopt unfamiliar eating habits.

Imposing a progressive cost constraint, exclusive of nutritional considerations, led to a low-cost energy-dense diet (41). The consumption of vegetables and fruit was low, and dietary energy was primarily provided by cereals and added fats. In contrast, deliberately increasing energy density of the diet did not lead to a major decline in diet costs (41). Figure 5 shows that the impact of cost on energy density (left panel) was much greater than the impact of energy density on cost (right panel).

In other words, deliberately selecting an energy-dense diet need not lead to lower diet costs. Conversely, restricting food expenditures will inevitably lead to more energy-dense diets. Consumers on a limited budget will find it difficult to find healthier diets unless they are willing to adopt unfamiliar eating habits, depart from social norms, and subsist on unpalatable foods. Strategies for dietary change, including the USDA Thrifty Food Plan (71), generally assume that the low-income consumers will do just that (78). Whereas good nutrition in the form of liver, dry legumes, peanuts, and canned fish can be inexpensive, such a diet scores low on taste, variety, enjoyment, and convenience. Although healthy diets can be assembled using inexpensive products (79, 80), USDA researchers acknowledge that this "may require some sacrifices in taste" (48). Persons facing economic constraints will preferentially select lower-cost energy-dense diets rather than abandon their usual eating habits. Strategies for dietary change ought to take food preferences and the usual eating habits into account.

Additional support for a causal link between poverty and obesity is provided by the growing price gap between healthy and unhealthy foods. Analyses of price increases during the period 1985–2000, shown in Figure 6, show that the cost of sweets, fats, and caloric beverages fell substantially in relation to fresh vegetables and fruit. Whereas the retail price of fresh vegetables and fruit registered a 120% increase, food items that best held their price were fats and sweets. If anything, these trends accentuate income-based disparities in the access to healthy diets.

Such disparities may not be remedied by small shifts in either incomes or in food prices. A recent USDA study showed that low-income households spent approximately $1.43 less per person per week on fruit and vegetables compared with higher-income households (81). Whereas higher-income households

![FIGURE 5](image_url)

**FIGURE 5.** Left, Impact of a cost constraint on dietary energy density in a linear programming model. Right, Impact of an energy density constraint on the cost of diets in a linear programming model. Printed with permission from reference 41.

![FIGURE 6](image_url)

did increase fruit and vegetable consumption after an increase in income, lower income households did not. One interpretation is that fruit and vegetables were not a priority and that low-income households chose to spend their limited resources on items that were perceived as more essential such as meat, clothing, or rent (81).

Americans have the lowest-cost food supply in the world and spend the lowest proportion of disposable income on food (~12%) (82). Until recently, no one has seriously questioned whether a low-cost food supply brought anything but benefits to the United States. However, studies are beginning to link the low cost of foods with the obesity epidemic. One study found that technological advances led to a decline in the price of food, which in turn led to higher energy intakes (83). The drop in food prices was said to account for up to 40% of the increase in body mass index since 1980 (83). Another study, based on national Behavioral Risk Factor Surveillance System data, linked higher obesity rates to lower food prices, a growing number of restaurants, and the higher cost of cigarettes (24). However, not all food prices have dropped. The downward trend in food prices, relative to other goods, was most marked for energy-dense foods, added sugar, and added fat.

To close the price gap between healthy and unhealthy foods, many policy options now call for taxes and levies on snacks, fats, and sweets (30, 31). However, such measures are primarily aimed at the lower-income consumer and have been criticized as punitive. It is the population subgroups with least resources that are most vulnerable to the obesity epidemic.

**WHY POVERTY AND OBESITY ARE CAUSALLY LINKED**

The rates of obesity and type 2 diabetes in the United States and other industrialized countries follow a socioeconomic gradient, with highest rates observed among minorities and the poor (52, 84–88). At the individual level, obesity rates are linked to low incomes, low education, minority status, and a higher incidence of poverty (52, 84, 85, 89). At the environmental level, obesity rates were higher in lower-income neighborhoods, legislative districts, and low-income states (90). Although obesity rates have been increasing steadily in both sexes, at all ages, in all races, and at all educational levels (85, 91), highest rates continue to be observed among the most disadvantaged groups.

The Healthy People 2010 report (92) acknowledged that obesity rates were higher among adolescents from poor households relative to middle- and high-income households; among African American women relative to white women, and among low-income relative to the more affluent groups. However, the dietary behaviors of obese persons continue to be viewed through the prism of medicine, biology, and behavior. Cravings for energy-dense fats and sweets have been explained by neurotransmitter imbalance (44, 93, 94). Excess consumption of added sugars and fats has been explained using such concepts as satiety deficits and passive overeating (5, 43, 44, 95, 96). The consumption of sweets and desserts has been explained in terms of an addictive personality, stress, depression, and seeking comfort in familiar foods. The failure to adhere to healthy diets has been explained in terms of physical access to supermarkets and grocery stores, marketing and distribution of healthy foods, urban sprawl, and the time spent commuting to work (97).

The present hypothesis is that the observed links between poverty and obesity are primarily accounted for by purely economic variables (40). What refined grains, added sugars, and added fats have in common is their low energy cost. Diets of lower-income households provide cheap, concentrated energy from fat, sugar, cereals, potatoes, and meat products but offer little in the way of whole grains, vegetables, and fruit (98-100). Likewise, low-income consumers are more likely to be frequent users of fast-food as opposed to full-service restaurants and are more likely to live in areas with less physical access to healthier foods. It is well established that higher diet quality, as measured by the Healthy Eating Index, is associated with higher incomes, more education, and with lower rates of obesity and overweight (39, 101).

**OBESITY PREVENTION AND THE COST OF DIETS**

The observation that healthier diets are likely to cost more poses some problems for the current strategies for health promotion. Although recognizing higher obesity rates among the more disadvantaged groups, the Surgeon General’s Call to Action for obesity prevention called for more nutritious diets, including more vegetables and fruit (102). The National Institutes of Health Obesity Education Initiative advised obese patients to look for guavas, persimmons, star fruit, kiwi, and papaya in preference to bologna and American cheese (103). Dietary recommendations in the Healthy People 2010 report mentioned a healthful assortment of vegetables, fruit, whole grains, lean-fat milk products, and fish, lean meat, poultry, or beans (92). African American men have been the focus of a public awareness cancer prevention campaign that encourages them to consume nine servings of fruit and vegetables per day (104).

Seemingly, the issue of diet costs has not been a concern. However, studies conducted in Australia, Canada, and the European Union have found that healthier diets cost more. One United Kingdom study (38) found that vegetarian diets high in fruit and vegetables were associated with higher diet costs. The direct monetary cost of the diet was calculated using average national prices from the 1995 United Kingdom National Food Survey and the Tesco home shopping catalog (38). In Denmark, low-fat diets for children were associated with higher costs (105). In another French study, diets with a higher content of vitamins and minerals were associated with higher diet costs (106). These studies contrast with the prevailing United States view that healthful diets do not represent an increased financial burden to the consumer and may actually cost less (79, 80).

The view that all foods are equally affordable is challenged by some recent reports. Figure 7, based on the Val-de-Marne data set, shows that meat, vegetables, and fruit contributed more to diet cost than to dietary energy, whereas grains, fats, and sweets provided energy at a lower cost. Diets that replace starches and fats with isocaloric amounts of lean meats and fresh produce are likely to cost more. Indeed, the average cost of the Atkins diet was recently estimated at $14.27/d, whereas the South Beach Diet was estimated at $12.78/d (107). Those figures contrast with the estimated $4 per person per day that some low-income families spend on food.

**THE ECONOMICS OF OBESITY**

Food policy interventions at the national and international level may be the most promising approach to making healthy
foods affordable and accessible to all (1). The World Health Organization (1) stated that the key to maintaining healthy weight was an affordable supply of fresh nutrient-rich foods. Such access could be facilitated through a combination of agricultural subsidies, pricing policies, regulatory action, and consumer education. Such approaches involve a cooperation between governments, academia, and the food industry.

Total US expenditures on all foods and beverages were estimated at $900 billion in 2002 (108), the lowest-cost food supply in the world. Annual medical expenditures in the United States that can be attributed to obesity were estimated at $75 billion in 2003 (109). To save on medical costs, consumers are encouraged to select healthier and more nutrient-dense diets. However, an increase in daily food expenditures of as little as 75¢ per person per day would mean an added expense of some $80 billion per year in diet costs. It is unclear how these costs are to be absorbed by the consumer.

Obesity lawsuits draw heavily on the parallels made between the food and the tobacco industries (36). However, the aptness of this analogy may require more careful thought. Stemming the obesity epidemic cannot be separated from stemming the tide of poverty. Are the various sectors of the food, grocery, and restaurant business legally liable for providing low-income consumers with inexpensive foods? Or is it possible that the rising obesity rates reflect an increasingly unequal distribution of incomes and wealth (110)? Does the obesity problem lie with fast-food outlets and vending machines, or are there broader societal issues that have to do with the falling value of the minimum wage, the lack of health and family benefits, and declining neighborhood resources? These issues need to be addressed through a concerted program of environmental and policy interventions. There is growing evidence that obesity in America is a largely economic issue.

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