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Optimizing the cardiovascular outcomes of weight loss¹,²

Jennie Brand-Miller

The past 2 y have seen a steady stream of reports indicating that restriction or modification of carbohydrate intakes can favorably affect weight loss and cardiovascular disease (CVD) risk factors (1–6). The article by Ebbeling et al (7) in this issue of the Journal represents one more in favor of diets with a low glycemic index (GI) or glycemic load (GL). Serum triacylglycerol concentrations in young overweight adults with similar weight loss fell nearly twice as far with the ad libitum low-GL diet as with the energy-restricted low-fat diet, whereas concentrations of plasminogen activator inhibitor 1, an important measure of thrombogenicity, significantly worsened (ie, rose) in subjects who were following the energy-restricted low-fat diet. The study was small (n = 23) and from a group at Harvard that had published other studies on the same topic, but it was long-term (12 mo) and carefully carried out.

That lowering daylong glycemia, with or without weight loss, might improve CVD risk should not come as a surprise. Many intervention studies have tested the hypothesis that low-GI diets will improve not only glucose control but also lipid metabolism. Twenty years ago, Jenkins et al (8) showed in 3-way crossover studies that low-GI diets improve triacylglycerol and total cholesterol concentrations in hyperlipidemic subjects more than do conventional low-fat diets. Recently, Patel et al (9) showed that women with advanced CVD who were awaiting bypass surgery spent significantly fewer days in the hospital than did their counterparts who were following a conventional low-fat diet (7.1 and 9.5 d, respectively). Slowing the rate of carbohydrate absorption per se by using the α-glucosidase inhibitor acarbose was found to reduce cardiovascular events by ≈50% over 3 y in a large population with impaired glucose tolerance (IGT).

Long-term studies in animals have provided additional evidence that the GI itself, and not fiber intake or any other confounding factor, is important in relation to weight gain, body fat, and CVD risk. Animals fed diets differing only in the type of starch (high- or low-GI) gained body fat faster with the high-GI diet than with the low-GI diet (10). Even when fed to similar body weight, high-GI diet–fed rats have more body fat (71%), less lean body mass, and higher plasma triacylglycerol concentrations than low-GI diet–fed rats (11).

Large-scale observational studies show links—even in nondiabetic persons—between the presence of postchallenge hyperglycemia and an increased risk of chronic disease (12). In a meta-analysis of 39 prospective studies of nondiabetic cohorts, Levitan et al (13) found that groups with the highest 120-min postload glucose concentration had a 27% greater risk of CVD than did those with the lowest glucose concentrations, and the relative risk was higher in women than in men (1.56 and 1.23, respectively). Adjustment for traditional CVD risk factors attenuated but did not abolish the relation. Moreover, Liu et al (14) showed that average dietary GI and GL were also independent predictors of 10-y prospective CVD risk in US women. The latter study is particularly important, because it implies that postprandial glycemia induced by carbohydrate foods in everyday settings (and not glucose tolerance testing) is clinically relevant.

There has been fundamental progress in showing that glucose itself can directly damage vascular cells, by a variety of mechanisms. All of these mechanisms appear to reflect a single hyperglycemia-induced process of overproduction of superoxide by the mitochondrial electron-transport chain (15). Normal concentrations of glycemia such as those encountered during a standard meal have been shown to acutely decrease plasma antioxidant capacity, which reflects a significant oxidative stress. Moreover, the vascular endothelium is a prime target because endothelial cells, unlike many other cells in the body, are unable to regulate glucose transport across the cell membrane.

Taken together, intervention, observational, and experimental studies suggest that postprandial glycemia plays a greater role in CVD than is generally acknowledged, perhaps more so in women than in men. Because decreasing the intakes of total and saturated fat has been the goal of efforts to reduce the incidence of obesity and CVD, high-carbohydrate foods have been recommended, not so much because of their intrinsic nutritional merit, but because they fill the calorie space formerly occupied by fat. But one of the more subtle changes in the food supply over the past few decades has been the replacement of traditionally processed grains by more highly processed, high-GI cereal products. Less-processed foods are more likely to contain slowly digested carbohydrates because the starches and sugars remain closely embedded in the plant’s original botanical structure, surrounded by bran and other barriers that inhibit starch gelatinization. In contrast, modern methods of food production using finer flours, extrusion technology, and high temperatures and pressures increase starch gelatinization and thus the rate of digestion in vivo. Compared with sugars, high-GI starch foods receive little attention, and yet they have a greater capacity than do sugars to

¹ From the Human Nutrition Unit, School of Molecular and Microbial Biosciences, University of Sydney, Sydney, Australia.

² Address reprint requests to J Brand-Miller, School of Molecular and Microbial Biosciences (G08), University of Sydney, Sydney, NSW 2006, Australia. E-mail: j.brandmiller@mmb.usyd.edu.au.
increase the glycemic and insulinoergic potency of the whole diet.

Because the overweight are now the majority in most industrialized nations, we can no longer afford to direct dietary guidelines to just the “healthy” population. Moreover, we need efficacious guidelines that work in practice, not just in theory. During the past 2 decades, when low-fat diets and plenty of cereal foods were actively promoted, health trends were the opposite of those we would wish. Along with obesity, the diagnoses of type 2 diabetes and IGT have soared, “maturity-onset” diabetes is being diagnosed in children, and the metabolic or insulin resistance syndrome affects 1 in 4 adults. Even normal-weight individuals can have the metabolic syndrome and thus a higher risk of CVD. Diseases such as polycystic ovarian syndrome, nonalcoholic steatohepatitis, and fatty liver, which have their roots in insulin resistance, have also reached alarming proportions.

It must now be clear that the conventional low-fat diet (with no consideration of the nature of the starch) is not the ideal diet for most of the population. Dietary Guidelines for Americans 2005 (16) sensibly gives emphasis to increased consumption of whole grains rather than to refined grains. However, this is unlikely to improve daylong glycemia, because many so-called whole-grain breads and breakfast cereals produce as much postprandial glycemia as do their white-flour counterparts (17). Moreover, recommending whole-grain and high-fiber cereals is nothing new—nutritionists have been doing that for at least 50 y. A high proportion of the population will dismiss outright any suggestion of eating whole grains or whole meal. We urgently need nutrition messages that fire the imagination and encourage even unmotivated people to adopt effective dietary strategies that reduce the risk of chronic disease. In Australia and the United Kingdom, the GI has become a popular concept in its own right. The message that slowly digested carbohydrates can “keep you fuller for longer” is one that the general public, young and old, intuitively understands. Indeed, many people warm to a plan that helps keep blood sugar concentrations “under control.” Furthermore, as Ebbeling et al (7) point out, their ad libitum low-GI diet is less extreme and restrictive than is either a low-energy, low-fat diet or a low-carbohydrate diet, and it still produces better outcomes.

It may be argued that the evidence for a role of GI or GL in weight management and CVD prevention is still insufficient to justify the place of either in nutrition advice to the general public. We need to clarify whether reducing the GI of the diet by changing the type of carbohydrate (substituting low-GI sources of carbohydrate for high-GI sources) or by substituting protein or fat for carbohydrate [or a combination of all 3 alternatives as Ebbeling et al (7) did] will have different metabolic consequences. Nevertheless, we must also acknowledge the shortcomings of the conventional low-fat (ipso facto high-GI) diet currently advocated by public health agencies and must be prepared to entertain the idea that the GI might be a useful and appealing concept after all.

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B vitamins and cognitive function: do we need more and larger trials?1,2

Stein Emil Vollset and Per Magne Ueland

The failure to directly translate promising observational associations between disease and intakes or blood concentrations of vitamins into effective chemoprevention or clinical treatment modalities is not new. The first major disappointment came a decade ago after studies of β-carotene and lung cancer (1). Contrary to expectations, the large Alpha-Tocopherol Beta-Carotene Cancer Prevention Study and Carotene and Retinol Efficacy Trial showed that this provitamin may increase lung cancer rates and all-cause mortality. Also, a recent meta-analysis of cardiovascular disease trials showed that high doses of vitamin E could increase all-cause mortality (2).

There is a clear association between total homocysteine (tHcy) concentrations and cardiovascular disease, and a series of large randomized clinical trials in patients with coronary heart disease or stroke are ongoing and a few studies have been completed. In these trials, the effects of treatment with folic acid in combination with vitamin B-6 and vitamin B-12 were compared with those of placebo. A large study of stroke patients in the United States showed no beneficial effect of B vitamins (3). In patients undergoing coronary angioplasty, one trial showed a clear reduction in restenosis (4), whereas another trial showed an increase in restenosis (5) with B vitamin therapy.

On the positive side, solid evidence from randomized trials and intervention studies has shown that folic acid prevents neural tube defects (6). This knowledge provides the basis for recommendations that women take folic acid if they plan to become pregnant and early in pregnancy and is why food-fortification programs have been implemented in several countries in North and South America.

Observational studies, including a recent investigation in 2871 subjects (7), have consistently reported that an elevated concentration of tHcy in serum or plasma is a risk factor for dementia and impaired cognitive function (8). This association has been explained by neurotoxic effects of homocysteine or the ability of elevated homocysteine to cause vascular lesions. Alternatively, the effects are mediated by the impaired function of the B vitamins involved in homocysteine metabolism, including vitamin B-12, folate, and vitamin B-6. Impaired vitamin B-12 status is known to be prevalent among the elderly, and vitamin B-12 deficiency may cause severe myelopathy and also prominent mental symptoms and memory loss (9). Poor folate status has been associated with depression and dementia in the elderly, and folate metabolism is linked to a variety of neurochemical processes (10). Vitamin B-6 status declines with age, and low blood concentrations of vitamin B-6 have been associated with impaired cognitive function and Alzheimer disease. Such associations could be explained by the involvement of vitamin B-6 in the synthesis of several neurotransmitters (11).

However, recent Cochrane Library reviews of randomized trials with folic acid, vitamin B-12, and vitamin B-6 provide no evidence of any improvement of cognition or dementia (11–13). The Cochrane reviewers, however, concluded that the current evidence was based on small trials and that the research question was sufficiently important to motivate more randomized trials.

In an elegant study from Sweden in this issue of the Journal, Lewerin et al (14) failed to show any effect of a combination treatment with folic acid, vitamin B-6, and vitamin B-12 on cognitive function or movement performance tests among elderly community-dwelling men and women. They conducted a double-blind, placebo-controlled randomized trial with 126 subjects allocated to the intervention and 69 subjects allocated to placebo. Their study also had an observational component, which showed strong relations of plasma tHcy and serum methylmalonic acid (MMA) with movement and cognitive performance tests.

The Swedish authors used an impressive panel of tests that assessed movement and cognitive performance in elderly subjects. The authors noted different associations between tHcy and MMA and different aspects of movement and cognitive performance. However, multiple comparisons of independent variables in a limited number of subjects may produce chance findings. Therefore, one should be cautious when interpreting the data and associations in terms of specific biological mechanisms.

The authors discuss their results relative to those of the now retired professor Ranjit Kumar Chandra (15). Their failure to reproduce Chandra’s results is not surprising in light of the recent controversy surrounding his research, which has received considerable attention both in the media (16) and in prominent medical journals (17).

When should we stop pursuing a treatment option with more randomized trials? Neither previous meta-analyses nor the single trial published in this issue of the Journal found evidence that B

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1 From LOCUS for Homocysteine and Related Vitamins, University of Bergen, Norway.

2 Address reprint requests to SE Vollset, University of Bergen, Department of Public Health and Primary Health Care, Kalfarveien 31, N-5018 Bergen, Norway. E-mail: vollset@uib.no.
vitamin treatment improves cognitive function and dementia. Before we conclude that this treatment option should be abandoned, several other issues need to be addressed.

First, is the observational and mechanistic evidence sufficient to justify further trials? The rationale for the studies that have been conducted is solid. Strong associations have been established between tHcy, Alzheimer disease risk, and cognitive dysfunction in several high-quality epidemiologic studies (8). The suggested mechanisms are biologically plausible, as indicated above.

Second, were the previous trials adequately powered to study the effects of vitamins on the study outcomes? The Swedish investigators did not provide information on how their sample size was determined. Calculations of sample size are routinely carried out before a trial is conducted, and it is recommended that such calculations be reported together with the study results (18). Only large trials can show important but smaller therapeutic gains relative to placebo. It is to be anticipated that much larger trials than those published so far are needed to establish or dismiss the role of B vitamin therapy in this area.

Third, were the doses and duration of the vitamin supplementation adequate? In terms of metabolite response, the doses used in the Swedish study seemed adequate. However, the doses required to normalize central nervous functions may be different from those that reduce concentrations of tHcy or MMA in blood and could be related to both the duration of therapy and the extent of vitamin depletion. A short-term trial may fail to show both efficacy and side effects. We suggest that future trials consider treatment over several years. Ideally, treatments should be tested as preventive measures and in persons with various degrees of functional impairment.

Fourth, designing the trial as a 2 × 2 or higher-order factorial trial might allow the investigators to gain additional information on the treatment efficacy of other vitamins or drugs without increasing cost. Such a design also allows the use of a commercial arm and a noncommercial (vitamin) arm and cofunding with industry. With scarce public resources and little commercial incentive to pursue research on vitamins, such a strategy—which has been used successfully in studies such as the Heart Protection Study (19)—might be the only way to fund needed future large vitamin trials.

Finally, because safety issues concerning the use of folic acid have been raised recently (20, 21), we suggest that future trials involving treatment over several years include monitoring of cancer outcomes.

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Aging muscle\textsuperscript{1–5}

K Sreekumaran Nair

ABSTRACT

Aging causes structural and functional changes in skeletal muscle in a wide range of species, including humans. Muscle changes in humans start in the fourth decade of life and cause frailty and disabilities. Associated changes in body composition form the basis of many metabolic disorders, such as insulin resistance, type 2 diabetes, hypertension, and hyperlipidemia, which result in an increased incidence of cardiovascular death. Decreases in the synthesis rates of many muscle proteins, specifically of myosin heavy chain and mitochondrial proteins, occur with age. The underlying causes of the reduction in mitochondrial biogenesis and ATP production seem to be decreases in mitochondrial DNA and messenger RNA. Reduced ATP production could be the basis of reduced muscle protein turnover, which requires energy. Both aerobic exercise and resistance exercise enhance muscle protein synthesis and mitochondrial biogenesis. Insulin and amino acids have also been shown to enhance muscle mitochondrial biogenesis and mitochondrial protein synthesis. However, the insulin-induced increase in muscle mitochondrial ATP production is defective in type 2 diabetic patients with insulin resistance. Moreover, a dissociation between increases in muscle mitochondrial biogenesis and insulin sensitivity after exercise has been noted in older persons. It remains to be determined whether muscle mitochondrial dysfunction causes or results from insulin resistance. Exercise seems to enhance the efficiency of muscle mitochondrial DNA in rodents. Reduced physical activity as a contributor of age-related mitochondrial dysfunction remains to be determined. It is proposed that a reduction in tissue mitochondrial ATP production signals the hypothalamic centers to reduce spontaneous physical activities. Voluntary physical activity is regulated by cognitive centers and could attenuate the progressive decline in mitochondrial functions that occurs with age. \textit{Am J Clin Nutr} 2005; 81:953–63.

KEY WORDS Aging, muscle, proteins, mitochondria, myosin, hypothalamus

INTRODUCTION

All species share the common destinies of birth, growth, aging, and death. We are born with a set of genes that largely determine our lives on the planet. The expression of genes is influenced by environmental factors, which can alter the rate of absolute growth, aging, and death. Environmental factors affect not only gene transcription but also gene translation (synthesis of proteins) and the posttranslational modification of proteins. The major determinants of alterations in body functions are proteins. Cellular functions are dependent on the ability to synthesize proteins with specific functions, interactions of proteins to proteins, proteins and genes, and proteins and metabolites. There are gradual and progressive alterations in body functions from birth to death. The changes are rapid during the growth phase, but the changes are rather slow during a rather prolonged period of aging—from \(\approx 30\) y to death. The aging process or changes related to secondary events or diseases cause rapid deterioration of body functions in most species in the last phase of life. The topic covered in this review is the aging of skeletal muscle; hypotheses that will hopefully stimulate new research in this area are discussed. Most of the presentation is based on results from our laboratory.

AGING IN HUMANS

Aging affects all species and substantially influences the scope of our activities and quality of life but what “aging” is exactly remains to be fully understood. The definition of aging is complicated by the occurrence of various diseases that modify body functions and tissue structures. The structural and functional changes related to diseases that are common in older persons are often hard to delineate from the aging process per se. Many disease processes and environmental factors profoundly influence the rate of aging. Moreover, the aging process occurs at different rates among different tissues, and the functional manifestations also vary. Aging-related changes in one organ might affect the functions of other organs. The aging process also differs substantially in different species. Aging in humans is vastly different from that in most other species because of the relatively long duration of life in humans after the genetic potential for growth is complete. All of these reasons make it difficult to study aging, and studies done in many other species cannot always be directly correlated to human aging.

\textsuperscript{1} From the Mayo Clinic College of Medicine, Division of Endocrinology and Endocrine Research, Rochester, MN.

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\textsuperscript{5} Address reprint requests to KS Nair, Mayo Clinic College of Medicine, Division of Endocrinology and Endocrine Research, 200 First Street SW, 5-194 Joseph, Rochester, MN 55905. E-mail: nair.sree@mayo.edu.

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The current review focuses on aging in muscle. For the purposes of this discussion, I assume that the aging process in humans starts from the fourth decade of life (at 30 y of age). This concept is supported by studies performed in human skeletal muscle (1–3), although it is not well established in all tissues. In healthy persons who died in motor vehicle accidents, it was observed that both muscle area and fiber numbers decreased from as early as the fourth decade of life (1, 4). We recently measured the cross-sectional area of muscle in the midthigh using computed tomography imaging in healthy human subjects ranging in age from 18 to 88 y. We observed an age-related decrease in muscle area starting from the fourth decade of life (2, 5) (Figure 1). In addition, a parallel decrease in muscle strength (knee extension) occurred with the decrease in muscle mass. We also noted that muscle strength normalized for fat-free mass in the leg also decreases with age, indicating that not only muscle mass declines with age but the efficiency of muscle mass (quality) does as well. Another key performance measurement that declines with age is maximal oxygen uptake (2), as noted in several previous studies (6, 7). It is a common belief that the muscle of older persons also fatigues faster than does that of young persons, although this has not been well documented by objective measurements. However, age-related changes vary substantially among persons, depending on their activity levels and other environmental factors.

It is likely that the decrease in muscle mass and muscle strength, in combination with reduced endurance, causes reduced physical activity (Figure 2). A reduction in muscle mass (responsible for ≈30% of resting energy expenditure and protein turnover as well as 70% body cell mass) and physical activity levels (contributes variable components to daily energy expenditure: 10–60%) decrease total energy expenditure in older persons. This reduction in overall energy expenditure in elderly persons results in an increased prevalence of obesity, especially abdominal fat accumulation. These alterations in body composition cause insulin resistance, which contributes to the development of a high prevalence of type 2 diabetes, hyperlipidemia, and hypertension in a genetically susceptible population (Figure 2). The combined effect of these metabolic abnormalities is increased cardiovascular death and other morbidities.

**MUSCLE CHANGES IN AGING**

Of interest, sarcopenia of aging is noted in a wide range of species, from nematodes, flies, rodents, and nonhuman primates to humans. A recent study in *Caenorhabditis elegans* showed gradual, progressive deterioration of muscle in this short-lived nematode that resembled human sarcopenia from midlife (8). The observed muscle deterioration occurred not only in body wall muscle but also in myofibrils of pharyngeal muscles. The sarcomeres of older *C. elegans* progressively became disorganized and contained fewer myosin thick filaments. Thus, it appears that *C. elegans*, like humans, undergo progressive loss of muscle mass and function. This nematode, which has a short life span of 20–25 d and whose genome is completely sequenced, is extensively studied to define the aging process. A remarkable finding of Herndon et al (8) was that these nematodes have a preserved central nervous system, even at an advanced age, and that muscle changes, which appear to occur at the beginning of midlife, indicate a dissociation between neuronal and muscle function.
changes. It is also fascinating that locomotor activity was found to be a predictor of life expectancy; those nematodes that showed earlier locomotor dysfunction had lower life expectancies. They found that mutation of \textit{age-1} (hx \textit{546}), which enhances locomotor activity in the aging population, delayed the age-related muscle nuclear changes. This finding suggests that the \textit{age-1} phosphatidylinositol-3-OH kinase might exert its effects on longevity in part by prolonging muscle integrity (8). Of note, an age-related decline in the synthesis rate of skeletal muscle myosin heavy chain (MHC) (Figure 3) has been reported in humans (1, 9-11) and may explain the decline in myosin content reported in \textit{C. elegans}. Because MHC is a key contractile protein, its reduction is likely to contribute to a decline in locomotor function.

The underlying mechanism that causes a decrease in muscle fibers, muscle mass, and muscle function—which are reported in human aging—has been extensively investigated. Many studies have reported an overall decrease in the skeletal muscle protein synthesis rate (2, 10, 12-14). Studies that did not normalize the activity levels and diets of the participants before measuring the fractional muscle protein synthesis rate failed to show any differences between young and older persons (15). There is substantial evidence that the muscle protein synthesis rate is responsive to exercise. In studies performed in young and old persons, resistance exercise stimulates the synthesis rate of mixed muscle proteins after 2 wk as well as it does after 3 mo, although the increment attenuates from 2 wk to 3 mo (16, 17). Resistance training for 3 mo also increased specific muscle proteins, such as MHC proteins, in older persons (10) (Figure 3). Studies have also shown that synthesis rates of mixed muscle proteins increase 3–4 h after resistance exercise (18). Mixed muscle protein synthesis has been shown to increase after 4 mo of aerobic exercise (2). Together, these results indicate that mixed muscle protein synthesis in both young and older persons responds to aerobic and resistance exercise programs. It is, however, important to appreciate that the effects of aerobic exercise and resistance exercise on muscle metabolism and muscle size are different. Aerobic exercise improves many metabolic functions, including insulin-induced glucose disposal and mitochondrial functions. It is likely

![Figure 3](https://example.com/figure3.png)
that the muscle functional changes induced by aerobic exercise are mediated by changes in specific proteins, especially the enzymes. Many of these proteins are likely to be of low concentration in muscle tissue and may not contribute much to the muscle mass. In contrast, resistance exercise mainly affects muscle strength and muscle mass, which may include many structural proteins. It is therefore likely that the proteins involved are different in response to resistance exercise from the aerobic exercise. Measurement of the fractional synthesis rate (FSR) of mixed muscle proteins represents an average of many proteins (low- and high-abundant proteins with low and high FSRs) and the changes in the FSR of mixed muscle protein synthesis rates may not translate into changes in muscle mass. Moreover, changes in muscle protein breakdown in response to different types of exercise also may occur at different rates for different muscle proteins. These issues are difficult to resolve but offer challenging opportunities for future research. Changes in the composition of meals or in substrate milieu have also been shown to affect muscle protein synthesis rates and protein turnover (19-21). The previous day’s meals can affect not only protein turnover but also insulin sensitivity (22), which in turn may affect muscle protein turnover. It is therefore critical to standardize the diet and exercise status in young and older persons before protein turnover studies to determine whether age per se has any effect on muscle protein turnover. Additional studies are needed to assess whether activity levels and diet have differential effects in young and older persons.

The important question then is: What causes the reduced baseline muscle protein synthesis rate? It is possible that younger persons are physically more active than are older persons, which by itself may affect muscle protein turnover. In our studies, we selected subjects who did not engage in any sport activities and did not deliberately exercise regularly. On the basis of a leisure-time activity questionnaire, the young and older subjects had similar activity levels. It remains to be determined whether younger persons are generally more physically active than are older persons on the basis of more objective measures and whether low activity levels in older persons caused the reduced muscle protein synthesis.

DOES AGE REDUCE ACTIVITY LEVELS?

There is substantial evidence from various species that activity levels decline with age (8, 23). Age-related declines in locomotor activity levels have been documented in C. elegans (8, 23), flies (24), and animals (25, 26). In humans, indirect measurements suggest an age-related decline in activity levels (27), and it is a common dogma that humans slow down with age. This general impression remains to be confirmed by reliable and objective measurements.

MITOCHONDRIAL DYSFUNCTION AS A DETERMINANT OF PHYSICAL ACTIVITY LEVELS

There is increasing evidence in rodents (28) and in humans (14) (Figure 4) that muscle mitochondrial dysfunction occurs with age. Changes in muscle mitochondrial function include decreases in mitochondrial DNA copy numbers, decreased mRNA concentrations in genes encoding muscle mitochondrial proteins (5, 28), reduced muscle mitochondrial oxidative enzyme activities, and reduced mitochondrial protein synthesis rates (5, 14) (Figure 4 and Figure 5). There are conflicting results on whether the actual muscle mitochondrial ATP production decreases with age (Table 1) (29-39). Our own preliminary results strongly indicate that there is an age-related decrease in muscle mitochondrial ATP production (40). A plausible hypothesis is that muscle mitochondrial ATP production is a determinant of physical activity levels (Figure 6). The rationale for the above hypothesis is that humans and other species need ATP for muscle contractile activities. The availability of ATP may signal, via an activity center, an alteration in activity levels. It is likely that the effects of mitochondrial ATP concentrations are spontaneous activities under the control of the hypothalamus. Evidence from animal studies supports the fact that activity levels are regulated by the hypothalamus, possibly in the paraventricular nucleus. Orexin A (hypocretin 1), when injected into the hypothalamic paraventricular nucleus, increased spontaneous activity levels (41) and food intake (42) in rodents. An intriguing possibility is that a neuropeptide that stimulates physical activity levels (needed for gathering food) also increases food intake. A recent review (43) outlines various catabolic neuronal pathways that reduce food intake and increase energy expenditure (eg, melanocortin neurons in hypothalamic arcuate nucleus) and are stimulated by leptin and insulin. In contrast, those anabolic pathways (eg, neurons containing neuropeptide Y) that enhance food intake and decrease energy expenditure appear to be inhibited by
insulin and leptin. It remains to be determined how these catabolic and anabolic regulatory centers are involved in the regulation of activity levels. Triiodothyronine administration increases activity levels in association with metabolic rate (44). It also remains to be determined whether neuronal or chemical mediators are involved in signaling hypothalamus from periphery to stimulate the center regulating activity levels. The different signals to the periphery from the activity regulating centers also remain to be determined. It is proposed that afferent sympathetic nerves signal the hypothalamic center (likely the paraventricular nucleus) about the status of muscle ATP production. The signal for changing activity level (spontaneous and possibly voluntary) is proposed to occur via efferent sympathetic nerves (Figure 6). It is also possible that the signals are mediated by chemicals.

Physical activity levels are likely regulated in 2 ways. Spontaneous physical activities are likely regulated by hypothalamic centers, and voluntary physical activities are regulated largely under cognitive control. It is proposed that spontaneous physical activity is regulated by hypothalamic centers in response to signals from the peripheral tissues, especially from skeletal muscle, and that spontaneous physical activity is reduced in older persons because of reduced muscle mitochondrial functions. The down-regulation of spontaneous activity may also influence the initiation of voluntary activities. Although voluntary activities are mostly under cognitive control, it is proposed that reduced spontaneous activity and other unknown regulatory factors interact with muscle mitochondrial functions to reduce the “motivation” of older persons to engage in voluntary physical activity. As a result, both spontaneous and voluntary physical activities decrease with age. Although it is a common dogma that humans “slow down” with age and are physically less active than are younger persons, hard supporting evidence is still not available.

EFFECT OF REDUCED PHYSICAL ACTIVITY LEVEL ON MUSCLE CHANGES

As discussed earlier in this review, synthesis rates of several muscle proteins are responsive to both aerobic and resistance exercise program (Figures 7 and 8) (2, 9). Substantial evidence indicates that mitochondrial biogenesis is stimulated by aerobic exercise (45). Muscle mitochondrial oxidative enzyme concentrations, such as cytochrome-c oxidase and citrate synthase, and mRNA concentrations of several genes encoding mitochondrial

![Figure 5](image-url)  
**Figure 5.** Progressive decrease in muscle mitochondrial messenger RNA concentrations of cytochrome-c oxidase subunit 4 (Cox 4) and NADH subunit 4 (ND4) in men and women from 20 to 80 y of age (5). AU, arbitrary units.

![Figure 6](image-url)  
**Figure 6.** Hypothesis for the regulation of physical activity. It is proposed that spontaneous activity levels are regulated by hypothalamic centers, possibly the paraventricular nucleus. Peripheral (mostly skeletal muscle) mitochondrial ATP send signals to the hypothalamus (afferent sympathetic nerve or chemical signal), which, via efferent sympathetic or chemical signals, up- or down-regulate activity levels. Voluntary activity levels are largely under cognitive control, although signals from hypothalamic centers may provide signals.

![Table 1](image-url)  
**Table 1.** Effect of age on muscle mitochondrial ATP production.

<table>
<thead>
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<th>Age effect</th>
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<tr>
<td>In vivo NMR studies</td>
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<td>Petersen et al, 2003 (29)</td>
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<td>Conley et al, 2000 (30)</td>
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<td>Chretien et al, 1998 (37)</td>
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<td>Barrientos et al, 1996 (38)</td>
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<td>Brierley et al, 1996 (39)</td>
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1↓, lower ATP production in older persons; ↔, no differences between young and old persons. NMR, nuclear magnetic resonance.
proteins and genes regulating mitochondrial transcription ([peroxisome proliferators–activated receptor co-activator 1 (PGC-1), nuclear respiratory factor 1 (NRF-1), and mitochondrial transcription factor A (TFAM)] are enhanced by 4 mo of aerobic exercise (2). It was previously shown that the master regulator of mitochondrial biogenesis is PGC-1, a protein localized in nucleus (46, 47). PGC-1, a cold-inducible co-activator of nuclear receptors, stimulates mitochondrial biogenesis and respiration in muscle cells and through regulation of NRF-1 and NRF-2. In addition, PGC-1 also binds to and coactivates the transcriptional function of NRF-1 on the promoter for TFAM. TFAM is a direct regulator of mitochondrial DNA replication and transcription (48). Overexpression of PGC-1 has been shown to enhance slow twitch muscle fibers in a mouse model (49), thus favoring the oxidative phenotype. We previously showed that aerobic exercise enhances the mRNA expression of PGC-1 in human skeletal muscle (2), thus favoring the oxidative phenotype. Moreover, as discussed earlier, in human skeletal muscle mRNA concentrations of NRF-1 and TFAM along with those of NADH subunit 4 (NADH 4) and cytochrome-\(\)c oxidase subunit 4 mRNA concentrations are enhanced by exercise programs (5). Long-term aerobic exercise seems to favor the phenotype of type I fibers (50) in skeletal muscle. It is clear that there is a common pathway for regulating the phenotype of oxidative muscle fiber type.

Mitochondrial biogenesis and mitochondrial DNA replication are stimulated by overexpression of a constitutively active form of calcium/calmodulin-dependent protein kinase IV (CaMK IV) (51). This effect of CaMK is mediated through induction of PGC-1 or expression (51). Chronic electrical stimulation of skeletal muscle causes a sustained elevation of intracellular calcium and activates calcium-regulated enzymes such as calcineurin and CaMK, both of which have been shown to synergistically activate slow and oxidative fiber-specific gene expression in myocytes (52). Muscle contractile activity through CaMK, AMPK, and calcineurin (51, 53–55) enhances mitochondrial biogenesis and oxidative phenotype of skeletal muscle. It appears that exercise favors oxidative muscle fiber type by well-defined regulatory pathways; CAMK and AMP kinase are key proteins in the cytoplasm of muscle cells that signal nuclear regulators involved in the biogenesis of mitochondria and expression of isoforms of MHC that determine the phenotype of muscle fibers. It remains to be determined whether this pathway of determining muscle phenotype is intact in aging. Future research may focus on the effect exercise on this regulatory pathway to determine the potential areas affected by aging.

Studies performed in rodents showed that mitochondrial DNA copy numbers are lower in skeletal muscle and liver (28) in older rats. The magnitude of the differences in mitochondrial copy numbers between young and older rats is related to the oxidative
Values represent the percentage of the average value in young rats greater in older animals in tissues such as the soleus muscle and liver. The lateral (gastroc. lat.) tissues. mRNA expression per DNA copy number was activity), than in gastrocnemius medial (gastroc. med.) and gastrocnemius liver (but not in the heart, which is continuously engaged in contractile activity), than in gastrocnemius medial (gastroc. med.) and gastrocnemius lateral (gastroc. lat.) tissues. mRNA expression per DNA copy number was greater in older animals in tissues such as the soleus muscle and liver. The values represent the percentage of the average value in young rats, Cox 1 and Cox 3, cytochrome-c oxidase subunits 1 and 3.

capacity of the tissues. For example, the lowering of mitochondrial copy numbers from young to older rats is greater in soleus (a highly oxidative tissue) than in gastrocnemius (Figure 9). However, DNA copy numbers in heart muscle are not different between young and older rats, despite the heart being a tissue with a high oxidative capacity. The potential explanation for this discrepancy is that heart muscle is in constant contractile activity and, as discussed in the previous paragraph, contractile activity may stimulate mitochondrial DNA replication and biogenesis through a CaMK-mediated pathway. Of note, in rodents, mRNA concentrations per DNA copy numbers are higher in the soleus of older rats than of younger rats (Figure 9). This intriguing observation suggests either an increased transcriptional efficiency (transcripts/DNA) or enhanced mRNA stability in older rats. If there is a higher transcription rate per DNA in older persons, a potential problem is the limit of the transcription rate, which may set the threshold for the ATP-producing capacity of DNA in a tissue. This is an intriguing possibility that requires further experimental evidence and may determine whether mitochondrial DNA copy numbers are a key determinant of the ATP-producing capacity of a tissue.

POTENTIAL MECHANISM OF AGING MUSCLE

The underlying mechanism of functional and structural changes in aging muscle remains to be fully understood. There is a generalized decline in muscle protein synthesis in humans with aging (2, 9, 10, 12, 14, 56). However, studies have shown that whereas the FSR of muscle mitochondrial protein and MHC (10) are lower in older persons, the synthesis rate of sarcoplasmic proteins is relatively higher in older persons, which suggests that the age-related inhibition of muscle protein is not a global effect on all proteins but is selective for certain proteins. Almost all of the previous studies were performed during the postabsorptive state, whereas muscle protein balance becomes positive only after a meal (19, 20). Studies to understand the regulation of postprandial protein dynamics are difficult to perform and are fraught with theoretical problems when interpreting the results. One potential approach is to label the proteins in a meal with stable isotopes and measure their incorporation into multiple muscle proteins. Advances in protein purification techniques and mass spectrometry will soon enable us to perform such studies. Studies have shown that milk proteins can be labeled with stable isotope of amino acids by infusing the isotopes in lactating cows (57). However, many studies using the intravenous infusion of substrates and insulin have been conducted. Amino acids alone (20) and insulin with glucose (19) achieve a positive protein balance across muscle bed. The main anticitabollic effect of insulin in the postabsorptive state is due to inhibition of muscle protein breakdown (19, 58), whereas the anticitabollic effect of amino acids is due to stimulation of protein synthesis and inhibition of protein breakdown (20). Both insulin and amino acids are largely responsible for protein accretion in skeletal muscle after a meal. Acute studies indicate that the main effect of insulin and amino acids is on the stimulation of muscle mitochondrial protein synthesis (59, 60) (Figure 10). Insulin and amino acids also enhance mRNA abundance in genes encoding mitochondrial proteins (Figure 11). It remains to be fully elucidated whether the transcriptional regulation of insulin and amino acids is selective for specific genes involved in specific functions. Insulin and amino acids have been shown to act via the signaling pathways (mainly by PI3 kinase and mTOR) to facilitate the initiation of the translation of messages in transcripts, thus promoting protein synthesis. The enzyme activities of selective mitochondrial enzymes and ATP production are also enhanced by insulin and amino acids (59) (Figure 11). It is possible that the effect of insulin may be secondary to other events, such as substrate oxidation or changes in substrate availability. The role of substrate oxidation, especially the role of free fatty acids and glucose on mitochondrial function, remains to be determined. In our studies, we replaced glucose during an insulin infusion.
achieving similar insulin concentrations) to prevent hypoglycemia and to maintain similar glucose concentrations in both diabetic and nondiabetic persons. The amount of glucose that was required to maintain similar blood glucose concentrations was lower in persons with type 2 diabetes. In type 2 diabetic patients, muscle mitochondrial ATP production was lower than in nondiabetic control subjects and insulin concentrations increased (59), although insulin concentrations were identical in both groups at low and high levels. The mechanism for the lack of stimulation of muscle mitochondrial ATP production in type 2 diabetic patients by insulin or amino acids remains to be determined. Reduced phosphorylation of signaling proteins and lower synthesis of mitochondrial proteins in type 2 diabetic patients may cause the lack of stimulation of muscle mitochondrial ATP production. Alternatively, a reduced delivery rate of glucose (and thus a reduced flux of glucose oxidation in muscle tissue) may affect muscle oxidative phosphorylation by an unknown mechanism. If insulin-induced signaling is important for muscle mitochondrial ATP production, insulin resistance in aging and type 2 diabetes could reduce muscle mitochondrial ATP production. Because insulin resistance occurs with aging, what remains to be determined is whether reduced insulin resistance causes a blunted increase in muscle mitochondrial ATP production after a meal or after an infusion of insulin with amino acids and glucose.

The reasons for the selective decrease in synthesis rates of certain muscle proteins in older persons also remain to be determined. The previous studies were based mainly on either the measurement of FSR of mixed muscle proteins (2, 13, 61) or selective fractions of muscle proteins, such as myofibrillar (56) or mitochondrial (14, 62) proteins. The FSRs of these mixed proteins represent the average of several proteins with a wide range of FSRs. Many of these proteins have different or even opposing functions. It is likely that, whereas aging inhibits the synthesis rate of some of these proteins, other proteins may not be affected by aging. In the case of mitochondrial proteins, the measurement of FSR of mixed proteins has definite functional importance because the main function of mitochondrial proteins is oxidative phosphorylation. However, >85% of these proteins (5 protein complexes with many subunits) are encoded by the nucleus, whereas others are encoded by mitochondrial genes. It is possible that if protein complexes involved at certain levels of electron transport are not available in sufficient quantities, ATP production may not occur. It is therefore important to study the abundances and FSRs of different mitochondrial proteins.

The interpretation of studies that show changes in muscle protein synthesis in response to interventions or in the basal steady state should be done with caution for a variety of reasons. One cannot assume that changes in the synthesis rate of a protein mixture can be translated to changes in the concentration of that protein mixture in the cell. First, a balance between protein synthesis and breakdown occurring in both the postabsorptive and postprandial states determines the change in protein content in a tissue. Second, the average synthesis rate of mixed muscle proteins measured in a tissue represents different proteins of a wide range of concentrations and turnover rates. For example, proteins that have a high turnover rate, such as enzymes, may make a larger contribution to the FSR but they make up a very small fraction of the concentration of mixed proteins. Proteins such as myosin, although constituting a major component of mixed muscle proteins, have a slow turnover rate and thus contribute only a small fraction to the synthesis rate of mixed muscle protein.

**FIGURE 11.** Effect of an infusion of insulin, while maintaining glucose and amino acid (AA) concentrations, on muscle mitochondrial ATP production (with the use of the substrates glutamate + malate and palmitoyl-L-carnitine + malate) and on ribosomal messenger RNA concentrations of genes encoding mitochondrial proteins [in the subunits NADH4 and cytochrome-c oxidase 3 and 4 (Cox 3 and Cox 4)]. *Significantly different from saline, P < 0.05. AU, arbitrary units.
Moreover, the concentration and synthesis rate of proteins such as myosin are unlikely to change during a short intervention period—at least most techniques are not sufficiently precise to detect the change. In contrast, many proteins with a fast turnover but in low concentrations may change during short interventions. Because proteins in relatively high concentrations (but low turnover rates), such as myosin, contribute more to muscle protein mass than do proteins in low concentrations (with fast turnover), the changes in synthesis rates of mixed proteins may not necessarily equate with changes in total protein mass. Arteriovenous balance studies are extremely useful for determining changes in the balance of total proteins in the muscle bed. However, these measurements are not precise because the measurements of many factors, such as blood flow, are known to be widely variable. Moreover, because these measurements have to be normalized for lean tissue mass or area in the leg or forearm, cross-sectional comparisons are fraught with many problems. Older persons have a greater proportion of fibrous tissues in muscle mass and less metabolically active tissue with a higher water content than do younger persons (63). As a result, the normalization of flux values per unit mass introduces further inaccuracy in cross-sectional comparisons.

The measurement of the synthesis rate of MHC (9, 10, 64, 65) is an advance over previous techniques that measure the synthesis rate of mixed proteins. However, even MHC exists in at least 3 isoforms in human muscle, and the relative compositions of these isoforms have a substantial effect on muscle phenotypes. When the MHC isoform 1 is predominant, it favors the phenotype of oxidative-fatigue-resistant slow twitch type 1 fibers; MHCIIa and IIx favor type IIa and type IIb fibers, respectively, which are fast twitch and glycolytic. Currently, no techniques are available to measure the synthesis rates of these isoforms or other key proteins in skeletal muscle. There are many promising approaches for purifying and measuring synthesis rates and concentrations of multiple muscle proteins in humans (66, 67). Application of these novel approaches may, in combination with mRNA measurements, DNA studies, and studies to determine regulation of both transcription and translation, are likely to provide a new understanding of the underlying mechanism of aging muscle.

SUMMARY AND CONCLUSIONS

Structural and functional changes in muscle during aging occur in a wide range of species, ranging from C. elegans to humans. The structural changes include a reduction in muscle mass and muscle fibers, and a shift of muscle fibers toward type 1 fibers. These structural changes are associated with muscle weakness, reduced endurance capacity, and insulin resistance. Muscle weakness is largely related to reduced mass but the muscle strength per unit mass of muscle also declines. A reduction in the synthesis rate of MHC, the key protein in the contractile apparatus, is likely to contribute to the muscle weakness. Myosin is deficient in the muscle of C. elegans, which reduces its locomotion. It remains to be determined whether the concentration of myosin and other key proteins involved in muscle contraction are reduced with aging in humans. In long-distance runners and in animal studies, type 1 fibers are rich in mitochondria and are relatively fatigue resistant. In contrast, the relative increase in type 1 fibers does not make older muscle fatigue resistant, perhaps because of a reduction in mitochondrial content with age. A reduction in mitochondrial ATP production could contribute to reduced endurance and muscle weakness. Increased mitochondrial DNA oxidative damage with aging and cumulative DNA damage could explain an overall reduction in mitochondrial DNA copy numbers in oxidative tissue, such as skeletal muscle (Figure 12) (68). A reduced mitochondrial DNA copy number may contribute to reduced mRNA abundance, which results in reduced mitochondrial protein synthesis and enzyme activity. The overall effect is a reduced capacity for oxidative phosphorylation. The reduced availability of ATP may contribute to an overall reduction in the remodeling process that involves the synthesis and breakdown of proteins, both of which are energy-consuming reactions in muscle.

Contractile muscle activity enhances muscle mitochondrial biogenesis through CaMK, AMP-activated kinase, and PGC-1α. Physical activity in the form of resistance and aerobic exercise stimulates muscle protein synthesis in both young and older persons. It remains to be determined whether different exercise programs have variable effects on different muscle proteins. Resistance exercise programs increase muscle mass; therefore, it is likely that the synthesis of structural proteins is enhanced by resistance exercise. In contrast, many metabolic changes occur with aerobic exercise with no increase in muscle mass. It is therefore likely that aerobic exercise stimulates the synthesis of many muscle proteins involved in metabolic processes. There is evidence in various species, from worms to rodents, that physical activity levels decrease with age. Although it is a common belief, supported by some experimental evidence, that physical activity levels decrease with age, further direct evidence is needed to verify this in humans. It is proposed that spontaneous activity levels in humans are regulated via the hypothalamus (possibly the paraventricular nucleus), and peripheral tissue mitochondrial ATP production is a determinant of hypothalamic control. In contrast, voluntary activities are likely to be mainly regulated by cognitive centers. Spontaneous activities decline with age in response to declining peripheral tissue mitochondrial function (Figure 13). This, together with a reduction in voluntary activities, further reduces mitochondrial biogenesis and functions as well as the synthesis rates of contractile proteins. Maintaining voluntary physical activities will partly prevent the age-related decline in muscle mitochondrial and contractile functions. Moreover, physical activities and related changes are also likely to delay or prevent insulin resistance. It remains to be determined whether age-related insulin resistance is due to or the cause of muscle mitochondrial dysfunction. It is likely that increases in

![Figure 12](image.png)
REFERENCES


33. Taylor DJ, Kemp GJ, Thompson CH, Radda GK. Ageing: effects on muscle mitochondrial function as a result of physical activity, and related changes, can prevent insulin resistance and related metabolic disorders that cause an increased incidence of cardiovascular deaths in elderly persons.

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There was no conflict of interest.

Aging Muscle

FIGURE 13. Hypothetical scheme for how mitochondrial damage and related changes can affect physical activity levels and how low activity levels can further reduce mitochondrial function. Reduced muscle function contributes further to metabolic disorders.
Physical activity energy expenditure predicts changes in body composition in middle-aged healthy whites: effect modification by age

Ulf Ekelund, Søren Brage, Paul W Franks, Susie Hennings, Sue Emms, Man-Yu Wong, and Nicholas J Wareham

ABSTRACT
Background: It is unclear whether physical activity energy expenditure (PAEE) predicts changes in body composition.

Objective: The objective was to describe the independent associations between PAEE and changes in body composition in a population-based cohort.

Design: This was a prospective population-based study conducted in 739 (311 men and 428 women) healthy middle-aged (median age: 53.8 y) whites. The median follow-up was 5.6 y. PAEE (MJ/d) was assessed by heart rate monitoring, individually calibrated by using the FLEX heart rate method. Fat mass (FM) and fat-free mass (FFM) were assessed by bioimpedance.

Results: Body weight (BW) at follow-up was significantly related to baseline PAEE (P < 0.05) after adjustment for sex, baseline age, FM, FFM, and follow-up time. A significant interaction between PAEE and age (P = 0.023) was observed. After the subjects were stratified (above and below the median for age), BW increased by a mean (± SD) of 1.7 ± 5.9 kg (P < 0.0001) in the younger cohort. In this group, follow-up FM was significantly associated with baseline PAEE (P = 0.036) after adjustment for confounders. In the older cohort, BW did not change between baseline and follow-up. In this group, in contrast with the younger population, follow-up BW, FM, and FFM were all significantly and positively associated with baseline PAEE (P < 0.01 for all).

Conclusions: Baseline PAEE predicts a change in FM in younger adults, who as a group gained weight in this study. In contrast, baseline PAEE in older adults—who were on average weight stable—is associated with a gain in BW, which was explained by an increase in FM and FFM. Am J Clin Nutr 2005;81:964–9.

KEY WORDS Energy expenditure, fat mass, fat-free mass, physical activity

INTRODUCTION

Obesity is multifactorial, involving genetic (1), social, cultural, and environmental (2) components. Gains in body weight (BW) and fat mass (FM) are the consequence of a positive energy balance over time. Thus, it has been suggested that the exponential rise in the prevalence of excess BW and body fat during past decades is most likely due to environmental changes, such as easy access to large portion sizes of energy-dense foods and reduced levels of physical activity energy expenditure (PAEE) or a combination of the 2 (2–4).

Previous population-based prospective studies that examined the association between physical activity and BW gain typically used subjective assessments of physical activity through self-report (5–11) and often also included self-reported height and weight (7, 9, 11). However, the measurement of physical activity in epidemiologic studies is difficult because of the complex nature of the exposure. Self-reported data on physical activity provides a relatively imprecise estimate of energy expenditure (EE) associated with physical activity (12). Many have argued that objective assessment methods of PAEE are needed to define the quantitative relation between activity and specific health outcomes, such as change in BW, and to characterize dose-response relations (12, 13).

Although BMI is widely used as a marker of overweight and obesity in epidemiologic studies (4), it does not allow separation of the components of FM and fat-free mass (FFM). Indeed neither the change in BMI nor in BW over time alone indicates whether change is due to an increase or decrease in FM or FFM or a change in both.

Therefore, the purpose of the present study was to describe whether objectively measured baseline PAEE predicts a change in BW after adjustment for potential confounders, including baseline FM and FFM. As a secondary aim, we examined whether baseline PAEE predicted change in body-composition variables (FM and FFM) after adjustment for confounding factors. This study was conducted over a 5-y period of follow-up in a randomly selected population-based cohort from the United Kingdom.

SUBJECTS AND METHODS

Study population

Participants were selected from the Medical Research Council Ely Study (14, 15), a prospective population-based cohort study...
of the etiology and pathogenesis of type 2 diabetes and related metabolic disorders. The volunteers were examined between 1994 and 1996 (baseline) and subsequently between 2001 and 2003 (follow-up). The median follow-up was 5.6 y. At baseline, 1120 healthy, middle-aged, white participants from the United Kingdom were assessed. Of these, 902 participants attended follow-up exams. A cohort of 739 participants (311 men and 428 women), in whom complete anthropometric and PAEE data were available, constitutes the sample for this report. All participants provided written informed consent. Ethical permission for the study was granted by the Cambridge Local Research Ethics Committee.

Repeated-measures substudy

During the year after the first assessment, a random subsample of 170 persons from the main cohort reattended our laboratories at 3 monthly intervals on 3 additional occasions for the reassessment of PAEE, REE, and body composition. The characteristics of the subsample and the methods used at reattendance, which did not differ significantly from those of the main study, were described in detail previously (15).

Body weight and body composition

Participants attended the laboratory after a 10-h overnight fast at baseline and follow-up. Height and BW were measured while the subjects were wearing indoor clothing with a rigid stadiometer and a calibrated scale. Resistance (Ω) was assessed by using a standard bioimpedance technique (Bodystat, Isle of Man, United Kingdom). This device was previously shown to be a valid (16) and reliable (17) measure of percentage body fat. Total body water and FFM were calculated by using the impedance index (height²/resistance), BW, and resistance according to the equations published by Sun et al (18). FM was calculated as BW minus FFM. Exactly the same procedures and equipment were used at baseline and at the follow-up visits.

Assessment of baseline PAEE

After the measurement of anthropometric factors, a standard protocol for individually calibrating HR and EE was undertaken. This method was described in detail elsewhere (15). The relation between EE (ie, oxygen consumption) and HR was assessed at rest, while the participants were lying and then sitting, with an oxygen analyzer calibrated daily by using 100% nitrogen and fresh air as standard gases (PK Morgan, Kent, United Kingdom). The participants then bicycled on a cycle ergometer at several different workloads to provide the slope and the intercept of the line relating EE to HR. Each subject cycled at 50 revolutions per minute, and the workload was progressively increased from 0 W to 37.5 W, 75 W, and 125 W in stages lasting 5 min each. At each workload, 3 separate readings were recorded of HR, minute volume, and expired air oxygen concentration. The 125-W level was only undertaken if the HR had not reached 120 beats per minute by the end of the 75-W level. The oxygen concentration in the expired air and minute volume data were used to calculate oxygen consumption after correction for standard temperature and pressure. EE (kJ/min) was calculated at each time point as oxygen consumption (L/min) × 20.35 (19). Mean REE was the average of the lying and sitting values. The slope and intercept of the least-squares regression line of the exercise points were calculated. Flex HR was calculated as the mean of the highest resting HR and the lowest HR while exercising. This point was used in the analysis of free-living minute-by-minute HR data to discriminate between rest and exercise. Below this point, EE was assumed to be equivalent to REE. EE above the flex point was predicted from the slope and intercept of the regression line calculated during the exercise test. Participants wore HR monitors (Polar Electro Ltd, Kemple, Finland) continuously during the waking hours over the following 4 d. HR readings were directly downloaded into a computer via a serial interface, and the individual calibration data were used to predict minute-by-minute EE for each person. PAEE (MJ/d) was calculated by subtracting REE from the minute-by-minute EE and thereafter averaged over the 4-d period and expressed in MJ/d.

Statistical analyses

The unadjusted means and SD of the means of anthropometric data and EE data were calculated. All data were analyzed in their continuous form, unless otherwise indicated. Nonnormally distributed variables were log transformed (ln) before analysis. Associations between PAEE and BW and FM at baseline and follow-up were assessed by correlation analyses. Using generalized linear modeling, the independent associations of PAEE with BW at follow-up were assessed, adjusting for sex, age, duration of follow-up, and baseline FM and FFM, and the regression coefficients for the exposure variables were presented. Because age was highly significant in this model, the interaction term PAEE × age was included in the model to test whether age modified the relation between PAEE and BW at follow-up. The purpose of doing this was to test whether age modified the relation between baseline PAEE and change in BW. A significant interaction effect was observed; therefore, we stratified the analyses above and below the median for age. To test whether change in BW is a function of change in FFM, FM, or both, we substituted BW with FM and FFM in subsequent models. In preliminary analyses, adjustments were also made for smoking and dietary fat intake at baseline, but, because these factors were not significant covariates and did not affect the relation between PAEE and body composition at follow-up, they were removed from the final models. All data were analyzed in their continuous form in the generalized linear models and corrected for error by using the method described below. PAEE was expressed by quartiles for illustrative purposes.

Statistical correction for measurement error

The within-individual and between-individual mean squares and the reliability coefficients for PAEE and body-composition variables were estimated by using the formulas described by Armstrong et al (20). We subsequently applied these error correction coefficients to the linear regression models constructed to test the association of PAEE with body-composition variables. The purpose of applying these error correction coefficients was to control statistically for measurement bias that occurs for exposure variables that are inherently highly variability, such as PAEE. Error correction coefficients were calculated under the assumption that the errors associated with repeated measures on the same individual were independent. The β coefficients are standardized to the variance in the exposure variable. Therefore, the magnitude of change in the outcome that is attributable to a 1-SD change in the exposure can be easily determined. We previously showed the utility of the method described by Armstrong
et al (20) in models designed to test the relative associations of physical activity with other metabolic phenotypes (15, 21).

RESULTS

The descriptive characteristics of participants at baseline and follow-up are displayed in Table 1. The median follow-up time was 5.6 y. BW was significantly higher in men than in women (P < 0.001) but did not change between baseline and follow-up. FM was significantly higher in women than in men (P < 0.001) and increased significantly between baseline and follow-up (P < 0.01). FFM was significantly higher in men than in women (P < 0.001) but did not change significantly between baseline and follow-up.

Baseline PAEE was significantly and inversely associated with age (r = −0.20, P < 0.001) at baseline. It was also inversely related to FM both at baseline (r = −0.14, P < 0.001) and at follow-up (r = −0.11, P < 0.001). Conversely, it was significantly and positively associated with BW (r = 0.31, P < 0.001; r = 0.30, P < 0.001) and FFM (r = 0.48, P < 0.001; r = 0.48, P < 0.001) at baseline and at follow-up, respectively.

The results of the generalized linear model for BW at follow-up, with baseline PAEE as the main exposure and with adjustment for sex, age, duration of follow-up, and baseline FM and FFM, are shown in Table 2. Age (P < 0.001) and baseline PAEE (P = 0.037) were significantly and negatively associated with BW at follow-up, whereas baseline FM and FFM (P < 0.0001) were positively associated with BW at follow-up. A significant interaction baseline PAEE and age (P = 0.023) was also observed.

We thereafter explored the association between baseline PAEE and FM at follow-up, with adjustment for sex, age, duration of follow-up, and baseline FM and FFM (Table 3). Baseline age (P < 0.0001) and baseline PAEE (P = 0.041) were significantly and negatively associated with FM at follow-up, whereas baseline FM was significantly and positively associated with FM at follow-up (P < 0.0001).

Because age modified the relation between baseline PAEE and BW at follow-up, we repeated our analyses in 2 age strata, above and below median baseline age (53.8 y). BW increased by 1.7 ± 5.9 kg (P < 0.001) in the younger cohort, in which BW at follow-up was significantly associated with baseline FM (β = 1.131, P < 0.0001), baseline FFM (β = 1.023, P < 0.0001), and baseline age (β = −0.185, P = 0.002) but not with baseline PAEE. FM at follow-up was significantly associated with baseline FM (β = 1.04, P < 0.0001), baseline FFM (β = 0.119, P = 0.023), and baseline PAEE (β = −0.001, P = 0.036). In the older cohort, there was no significant change in BW (0.05 ± 4.9 kg) between baseline and follow-up. BW, FM, and FFM at follow-up were all significantly and positively associated with baseline PAEE (β = 0.0018, P = 0.001; β = 0.00053, P = 0.007; and β = 0.0013, P = 0.001, respectively) after adjustment for sex, baseline age, duration of follow-up, and baseline FM and FFM. The relation between change in FM and quartiles of baseline

### Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men (n = 311)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>54.1 ± 10.3</td>
<td>59.7 ± 10.5</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>81.6 ± 11.3</td>
<td>82.2 ± 12.8</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>174.9 ± 6.7</td>
<td>174.5 ± 6.7</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>19.6 ± 5.7</td>
<td>20.8 ± 6.2</td>
</tr>
<tr>
<td>Fat-free mass (kg)</td>
<td>62.0 ± 7.5</td>
<td>61.4 ± 8.4</td>
</tr>
<tr>
<td>Women (n = 428)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>53.3 ± 10.1</td>
<td>59.0 ± 10.2</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>69.0 ± 13.0</td>
<td>70.1 ± 14.8</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>162.3 ± 6.1</td>
<td>161.9 ± 6.2</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>26.0 ± 8.9</td>
<td>27.5 ± 10.3</td>
</tr>
<tr>
<td>Fat-free mass (kg)</td>
<td>43.0 ± 5.9</td>
<td>42.6 ± 6.1</td>
</tr>
</tbody>
</table>

1 All values are x ± SD. No significant time-by-sex interactions were observed.
2,3 ANOVA for between-time differences: 2P < 0.001, 3P < 0.01.
4 ANOVA for between-sex differences, P < 0.001.

### Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>β</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>0.292</td>
<td>(−0.677, 2.26)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>−0.2062</td>
<td>(−0.287, −0.126)</td>
</tr>
<tr>
<td>Follow-up time</td>
<td>0.00171</td>
<td>(−0.00113, 0.00455)</td>
</tr>
<tr>
<td>Baseline FM (kg)</td>
<td>1.082</td>
<td>(1.018, 1.145)</td>
</tr>
<tr>
<td>Baseline FFM (kg)</td>
<td>0.9762</td>
<td>(0.898, 1.058)</td>
</tr>
<tr>
<td>PAEE (MJ/d)</td>
<td>−0.0037</td>
<td>(−0.00022, 0.00024)</td>
</tr>
<tr>
<td>PAEE × age</td>
<td>0.000078</td>
<td>(0.000013, 0.00014)</td>
</tr>
</tbody>
</table>

1 Data were obtained with the use of a general linear model (analysis of covariance). FM, fat mass; FFM, fat-free mass.
2 P < 0.001.
3 P < 0.05.

### Table 3

<table>
<thead>
<tr>
<th>Variable</th>
<th>β</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>−0.759</td>
<td>(−2.093, 0.574)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>−0.1713</td>
<td>(−0.226, −0.117)</td>
</tr>
<tr>
<td>Follow-up time</td>
<td>0.00183</td>
<td>(0.000091, 0.0038)</td>
</tr>
<tr>
<td>Baseline FM (kg)</td>
<td>1.034</td>
<td>(0.991, 1.077)</td>
</tr>
<tr>
<td>Baseline FFM (kg)</td>
<td>0.046</td>
<td>(−0.00024, 0.1)</td>
</tr>
<tr>
<td>PAEE (MJ/d)</td>
<td>−0.0024</td>
<td>(−0.000475, −0.000061)</td>
</tr>
<tr>
<td>PAEE × age</td>
<td>0.00004</td>
<td>(0.0000037, 0.000084)</td>
</tr>
</tbody>
</table>

1 Data were obtained with the use of a general linear model (analysis of covariance). FM, fat-free mass.
2 P < 0.001.
3 P < 0.05.
PAEE and the relation between change in FFM and quartiles of baseline PAEE are shown in Figure 1.

Subsequently, we applied bivariate error correction coefficients derived from the repeated-measures substudy to the models that included PAEE. In the younger cohort, baseline PAEE was significantly and inversely related to FM at follow-up (uncorrected standardized $\beta = -0.0205, P = 0.03$) and after multivariate error correction (standardized $\beta = -0.0358, P = 0.03$) and adjustment for the same confounding variables as above. In the older cohort, baseline PAEE (MJ/d) was significantly and positively associated with BW (uncorrected standardized $\beta = 0.0130$), FM (uncorrected standardized $\beta = 0.0128$), and FFM (uncorrected standardized $\beta = 0.0130$) and after multivariate error correction ($\beta = 0.02580, \beta = 0.02589,$ and $\beta = 0.02622$ for BW, FM, and FFM, respectively) at follow-up ($P < 0.001$ for all in the older cohort).

**DISCUSSION**

The nature of the longitudinal relation between physical activity and change in body composition is uncertain. This may be because attempts to assess this relation have used relatively imprecise self-reported measures of exposure and outcome variables. In this study we quantified the outcome and exposure variables objectively and observed independent inverse associations between PAEE and change in BW and FM over a 5.6-y period, after adjustment for confounding factors. However, the prospective relation between PAEE and BW was modified by age. In younger middle-aged adults, who on average gained weight during the follow-up period, baseline PAEE was inversely associated with change in BW but was not associated with the change in BW and FFM. In contrast, older middle-aged adults, who on average were weight stable over the follow-up period, baseline PAEE was positively associated with an increase in BW, FM, and FFM. Adjustment for the effect of measurement error substantially strengthened these associations.

Prospective analyses have suggested that physical activity, assessed through self-report, prevents weight gain (8-10). Other studies have only observed a significant association between weight gain and self-reported physical activity at follow-up (5–7), whereas others have observed no significant association between activity and change in BW (22, 23). Furthermore, sex differences for the longitudinal association between self-reported physical activity and change in BW and composition have been observed (8, 11). It was recently suggested that physical inactivity was not associated with the development of obesity, whereas obesity may lead to inactivity (24).

Prospective studies (25–27) using objective measurements of EE with the doubly labeled water method have failed to show a longitudinal association between baseline PAEE and change in FM. However, these studies were performed in relatively small and select samples, and 2 of these studies were conducted in children (26, 27). A decrease in REE over the follow-up period, without a subsequent change in PAEE, may explain our observation that PAEE predicted FM gain in the younger cohort. However, we did not observe a significant difference in REE between periods (data not shown), which indicated that this cannot explain our findings.

Although we observed a statistically significant association between baseline PAEE and FM in the younger adult cohort, the clinical significance of this finding may be questionable given the weak associations and the small amount of variance explained in the outcome attributable to PAEE (<1%). Because even the most active quartile in the younger cohort gained FM over the measurement period, it is tempting to speculate that energy intake may play a greater role than PAEE in the gain in FM and the pathogenesis of obesity. In support of this hypothesis, a recent prospective study showed that energy intake but not EE attributable to physical activity was related to BW gain in adult Pima Indians (25). The gain in BW in younger middle-aged adults of $\approx 1.7$ kg over the follow-up period equates to a positive energy balance of $<30$ kJ/d, or a value equivalent to the energy cost of $\approx 2$ min of brisk walking per day. Such a small energy imbalance cannot be quantified with current methods, and it seems unlikely that the issue regarding energy intake compared with EE in the development of obesity can be resolved at this time.

In contrast with our observation in the younger adult cohort, the older group remained weight stable over the follow-up period. In this group, we observed a statistically significant positive association between baseline PAEE and gain in BW, which was explained by an increase in FM and FFM. Aging is associated with weight loss and sarcopenia, and it was recently suggested that physical activity attenuated weight loss in subjects older than 65 y of age (28). Furthermore, it was shown that low FFM can be increased by physical activity in the elderly (8). Our findings partly support this and also suggest that high levels of physical activity are associated with an increase in BW. The ideal BW for
older people is controversial (29, 30). However a relatively high BMI (≥27) is associated with minimum health hazards in the elderly (30). Furthermore, because weight loss is strongly associated with deleterious health in older people (31, 32), our data support the role of physical activity for the prevention of weight loss and improved health at older ages.

As in all observational studies, our data only suggest a causal association between baseline PAEE and body composition. However, it is unlikely that these associations are due to chance. First, the ability to detect the association between PAEE and change in body–composition components that we report in the present study is dependent on several factors. These factors include the precision of both exposure and outcome measurements, the sample size, and the difference in the magnitude of the association between exposure and outcome. The present study was undertaken in a large randomly selected population-based cohort in whom objective assessments of EE are available. PAEE was assessed through individually calibrated heart rate against REE and exercising EE. Our measure of PAEE has been shown to correlate well with PAEE measured with the doubly labeled water method (33), which is considered by many to be the gold standard method for assessing free-living EE. Moreover, the heart rate flex method is considerably more reliable and valid than subjective techniques such as questionnaire and interview (34). Furthermore, when we repeated our analyses with adjustment for exposure measurement error (35), the observed associations were considerably strengthened, which provides additional evidence that our measure of PAEE is valid and reliable. Our measure of body composition was obtained by bioimpedance, a method less precise than underwater weighting or dual-energy X-ray absorptiometry. However, because the same method was used at baseline and follow-up, it is unlikely that the measure of body composition was differentially biased between exams. Additionally, the direction of the associations between baseline PAEE and change in BW (which is measured with high precision) in the 2 age groups was similar to the associations between baseline PAEE and change in body fat.

In summary, PAEE predicts change in FM in younger adults, who on average gain weight over 5 y. In contrast, in older adults, who remain relatively weight stable, PAEE is associated with a gain in BW that is attributable to an increase in FM and FFM. Our results underscore the importance of developing physical activity programs designed to prevent obesity in younger middle-aged adults who are likely to gain weight. In contrast, such programs may prevent weight loss and preserve FFM in older adults who are weight stable.

UE conceived the hypothesis for this manuscript, performed the data analyses, and drafted the manuscript. SB and PWF provided critical input for the conception of this manuscript and assisted with the editing of the manuscript. SE and SH were the lead field epidemiologists on the project and were primarily responsible for the data collection and helped with the editing of the manuscript. SB, PWF, and NJW provided critical input for the data analyses and on the previous versions of the manuscript. NJW was the principal investigator of the Medical Research Council Ely Study and was responsible for the overall study design. All authors took part in the discussion of the results and approved the final version of the manuscript. None of the authors had any conflicts of interest.

REFERENCES


Obesity and risk factors for the metabolic syndrome among low-income, urban, African American schoolchildren: the rule rather than the exception?1–3

Carol L Braunschweig, Sandra Gomez, Huifang Liang, Kristin Tomey, Bethany Doerfler, Youfa Wang, Chris Beebe, and Rebecca Lipton

ABSTRACT

Background: Adult obesity is associated with the metabolic syndrome; however, the prevalence of the metabolic syndrome among young children has not been reported. Clinic-based screening efforts for the metabolic syndrome in low-income neighborhoods, where obesity is prevalent, are limited by minimal health insurance coverage and inadequate access to health care. School-based obesity screening programs may effectively target high-risk populations.

Objective: The objective was to describe the prevalence of overweight and features of the metabolic syndrome (defined as the presence of ≥3 of the following risk factors: HDL ≤ 40 mg/dL, triacylglycerol ≥ 110 mg/dL, and blood pressure or waist circumference at or above the 90th percentile) in a pilot, school-based screening program.

Design: A cross-sectional study of obesity and the metabolic syndrome was conducted in third- to sixth-grade, low-income, urban, African American children. Lipid and glucose concentrations were measured in fasting capillary finger-stick samples.

Results: Age-and sex-specific BMI percentiles were assessed in 385 students, 90 of whom were full participants in this study (participants) and 295 of whom had only height and weight measurements taken (other students). Risk factors of the metabolic syndrome were assessed in the 90 participants (23%). No significant differences in BMI percentiles were found between the participants and the other students. Overall, 44% of the participants had BMIs at or above the 85th percentile, and 59% had an elevated BMI or one metabolic syndrome risk factor. The metabolic syndrome was present in 5.6% of all participants, in 13.8% of participants with BMIs at or above the 95th percentile, and in 0% of participants with BMIs below the 95th percentile.

Conclusions: Most of the African American children attending 2 urban schools in low-income neighborhoods were overweight or had one or more risk factors for the metabolic syndrome. School-based screening programs in high-risk populations may provide an efficient venue for the screening of obesity and related risk factors.

INTRODUCTION

In the United States, the prevalence of overweight among children—defined as a body mass index (BMI; weight in kilograms divided by height squared in meters) at or above the 95th percentile on age- and sex-specific growth charts—has tripled in the past 30 y (1–3). Current data indicate that 15% of US children between 6 and 11 y of age are overweight, and nearly 20% of non-Hispanic black children are overweight. Obesity in childhood is a public health concern because it increases the risk of developing hypertension, elevated cholesterol, type 2 diabetes, coronary heart disease, orthopedic disorders, and respiratory disease (4–6); has a significant negative effect on childhood emotional development (7, 8); has been shown to track to adult obesity; and increases the risk of adult mortality (9–11).

Insulin resistance and its associated metabolic characteristics have been proposed as a link between obesity and disease. The metabolic syndrome is a constellation of metabolic abnormalities aligned with insulin resistance that predicts premature coronary artery disease and type 2 diabetes; it affects ≈23% of the US adult population (12, 13). Among adolescents (12–19 y), Cook et al (14) defined this syndrome as the presence of ≥3 of the following risk factors: triacylglycerols > 110 mg/dL, HDL cholesterol ≤ 40 mg/dL, waist circumference (WC) at or above the 90th percentile, fasting glucose ≥ 110 mg/dL, and blood pressure (BP) at or above the 90th percentile. Using this definition to analyze data from the third National Health and Nutrition Examination Survey (NHANES III, 1988–1994), they estimated the overall prevalence among adolescents to be 4%. According to the National Cholesterol Education Program, the increasing rate of obesity and its association with insulin resistance and type 2 diabetes positions the metabolic syndrome to play a greater role in premature cardiac disease than tobacco use (12). To our knowledge, estimates for the metabolic syndrome among younger children have not been described, possibly because NHANES does not include the biochemical data needed for children aged <12 y.

Given the effect of obesity on a vast array of diseases that track into adulthood, efforts aimed at early detection, treatment, and monitoring of its risk factors are needed. Results from NHANES

1 From the Department of Human Nutrition, University of Illinois at Chicago, Chicago, IL.
2 Supported by Takeda Pharmaceuticals America.
3 Address reprint requests to CL Braunschweig, Department of Human Nutrition, 1919 West Taylor Street, University of Illinois at Chicago, Chicago, IL 60612. E-mail: braunsch@uic.edu.

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1999–2000 suggest that low-income, African American grade school children likely have greater prevalence rates of obesity and elevated lipid and glucose concentrations and BP than the national average; however, studies in this population are lacking. Clinic-based screening programs in low-income neighborhoods, where these characteristics are highly prevalent, are plagued by minimal health insurance coverage, which limits routine access to health care. Detecting risk in individuals who might not otherwise be assessed is one of the objectives set forth by the National Heart Lung and Blood Institute and the American Heart Association for public cholesterol screening (15). Additionally, a small but growing body of literature suggests that the treatment of obesity tends to be underrecognized and undertreated by physicians, particularly in minority and low-income environments (16–18). A school-based universal health screening program that targets students in low-income minority neighborhoods may provide an optimal environment for early detection and surveillance of obesity and related risk factors.

The purpose of this study was to describe a pilot, school-based screening program in urban, low-socioeconomic, third- to sixth-grade African American schoolchildren for obesity and features of the “presumed” metabolic syndrome.

**SUBJECTS AND METHODS**

**Subjects**

All children in grades 3–6 enrolled in the 2002 academic year at 2 public elementary schools in Chicago who had their sex- and age-specific BMIs assessed as part of a school-wide health report card were eligible for participation. Letters inviting interested parents and guardians to provide phone numbers were mailed to the student’s homes. Those who responded were given detailed information about the research project and participation requirements. The study protocol was approved by the Ethics Committee of the Institutional Review Board of the University of Illinois at Chicago. Informed consent was obtained from all parents or guardians, and participating students signed an assent agreement.

**Methods**

Data collection occurred before the start of the school day. Height and weight measurements were rotated and performed twice for each participant with the use of standardized, recommended techniques (19, 20). Participants were classified as underweight (<5th percentile), normal weight (≥5th and <85th percentile), at risk of overweight (≥85th and <95th percentile), or overweight (≥95th percentile) according to the 2000 Centers for Disease Control and Prevention growth charts (2). The WC measurement was made at the narrowest observed point between the bottom of the rib cage and the umbilicus; if a point of least circumference was not apparent, measurements were obtained at the umbilicus to the nearest 0.1 cm. Measurements were made by using an inelastic 3/8-in (≈1 cm) measuring tape that was calibrated with a metal tape measure on a monthly basis (19). A WC at or above the 90th percentile for age, ethnicity, and sex according to the 2000 Centers for Disease Control and Prevention growth charts (2) was categorized as a risk factor for the metabolic syndrome.

BP measurements were taken twice, after the participant sat comfortably for 5 min, with an appropriately sized cuff on the right arm, which was slightly flexed at heart level. The second BP measurement was used for analysis (21). A systolic or diastolic BP at or above the 90th percentile on the basis of normative BP tables (22) for height, age, and sex was considered a risk factor for the metabolic syndrome.

Fasting finger-stick capillary samples were used to assess total cholesterol, LDL cholesterol, HDL cholesterol, triacylglycerols, glucose, and hemoglobin A1C (Hb A1c). The lipid and glucose concentrations were analyzed by using the Cholestech LDX Lipid Monitoring System (Cholestech Corp, Hayward, CA). This system correctly classified fasting individuals into the appropriate National Cholesterol and Education Program risk groups ≥95% of the time (23); and further, the CVs for between- and within-runs were higher than the National Cholesterol and Education Program standards for accuracy and precision (manufacturer’s information). Hb A1c was analyzed with the use of the DCA 2000 + Analyzer (Bayer Healthcare, Diagnostics Division, Tarrytown, NY). The DCA 2000 analyzer has a within-run reliability intraclass correlation CV of 2.8% and a between-run correlation coefficient of 0.996 (24). Quality control procedures for calibration of the Cholestech LDX and DCA 2000 were performed according to manufacturer guidelines and were recorded daily. Lipid concentrations were categorized on the basis of American Heart Association guidelines for children (25).

The metabolic syndrome was defined as the presence of ≥3 risk factors according to the guidelines developed by Cook et al (14) for adolescents. The 1998 guidelines of the American Academy of Pediatrics were used for the lipid guidelines (26). The criteria used for both lipid concentrations and metabolic syndrome are provided in Table 1. In November 2003, the American Diabetes Association changed the glucose categorization for impaired fasting glucose (IFG) from ≥110 mg/dL to ≥100 mg/dL (27). Despite this change, we retained the earlier cutoff guideline.

**TABLE 1**

<table>
<thead>
<tr>
<th>Criteria for lipid concentrations and the metabolic syndrome</th>
<th>Lipids</th>
<th>Metabolic syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>Acceptable: &lt;170</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Borderline: 170–199</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>High: ≥200</td>
<td>NA</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>Acceptable: &lt;110</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Borderline: 110–130</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>High: ≥130</td>
<td>NA</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>Acceptable: &gt;35</td>
<td>≤40</td>
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<tr>
<td></td>
<td>Low: &lt;35</td>
<td>≥40</td>
</tr>
<tr>
<td></td>
<td>High: ≥150</td>
<td>≥110</td>
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<tr>
<td>Triacylglycerols (mg/dL)</td>
<td>Desirable: 80–109</td>
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<td>Impaired fasting glucose: 110–125</td>
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<td>Diabetes: ≥126</td>
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<tr>
<td>Blood pressure (mm Hg)</td>
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<tr>
<td>Waist circumference (cm)</td>
<td>NA</td>
<td>≥90th percentile</td>
</tr>
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</table>

1 NA, not applicable.

2 Defined as the presence of ≥3 of the listed risk factors.
TABLE 2
Age, height, weight, and BMI of the participants and the other third- to sixth-grade students

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
<th>BMI percentile</th>
<th>BMI percentile category [% (n)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;5th (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5th to &lt;85th (55.6 (50))</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>85th to &lt;95th (21.1 (19))</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>≥95th (23.3 (21))</td>
</tr>
</tbody>
</table>

Participants (n = 90) | Other students (n = 295)

± SD | Median (range) | ± SD | Median (range)

Age 10.5 ± 1.3 | 10.5 (8.3–12.9) | 11.0 ± 1.7 | 10.8 (8.0–14.9)
Height 145.0 ± 11.2 | 143.6 (118.7–171.8) | 146.6 ± 12.6 | 147.1 (119.2–186.9)
Weight 44.1 ± 14.7 | 40.9 (22.6–92.1) | 46.7 ± 19.8 | 42.4 (20.5–124.0)
BMI 20.5 ± 4.9 | 19.3 (14.2–38.2) | 20.9 ± 5.4 | 19.4 (14.2–47.2)
BMI percentile 70.8 ± 27.4 | 81.4 (8.9–99.6) | 70.6 ± 26.7 | 77.2 (2.6–99.9)
BMI percentile category [% (n)]
<5th 0 | 1.4 (4)
5th to <85th 55.6 (50) | 57.3 (168)
85th to <95th 21.1 (19) | 19.3 (57)
≥95th 23.3 (21) | 22.4 (66)

1 No significant differences were found.

for IFG of ≥110 mg/dL to allow for comparisons between our population and that of Cook et al (14) and because, to our knowledge, it remains the only study that has defined the prevalence of the metabolic syndrome in a nationally representative sample of children. Because we used the more stringent cutoff, our estimates for elevated glucose should be viewed as a conservative estimate for the prevalence of elevated glucose. Glucose concentrations >125 mg/dL were categorized as indicating diabetes.

All participants received a growth chart with their age- and sex-specific BMI printed on it, a description of how to interpret their measurement, and a phone number for discussion of their results with the research team. They also received a copy of their BP, lipid, and glucose profiles. If a measured value was outside the desirable range, it was repeated. For analysis purposes, the lower of the 2 measurements was used. Participants with concentrations that persisted outside the normal range were provided referral information for free medical evaluation at a neighborhood health clinic as well as individualized diet counseling by the research dietitians (for both parents and children).

Statistical analysis
Standard descriptive statistics (mean, median, range, and SD) were assessed for continuous variables. Nonnormally distributed variables were transformed as necessary. Chi-square tests were used to examine whether the participants’ distributions within the 4 BMI categories (<5th percentile, 5th to <85th percentile, 85th to 95th percentile, and ≥95th percentile) were similar to those of the other third- to sixth-grade students. Wilcoxon’s rank-sum test was used to determine whether the participants’ weights and BMIs were similar to those of the other third- to sixth-grade students. Student’s t test was used to assess differences in age and height of the participants and other third- to sixth-grade students and to detect differences in risk factors between sexes. Prevalence and 95% CIs for individual features of the metabolic syndrome and different numbers of risk factors were calculated and used to assess differences in the prevalence of metabolic syndrome by sex and BMI. All data were entered and cleaned in EPIINFO 6.0 (1997; Centers for Disease Control and Prevention, Atlanta), and statistical analyses were performed by using SAS version 8.2 (SAS Institute, Cary, NC).

RESULTS
Overall, 23% (n = 90) of the 385 eligible students assessed for age- and sex-specific BMI measurements were recruited. All participants were African American and qualified for free school lunch; 28% had private medical insurance, 64% had Medicaid, and 8% had no insurance. Age- and sex-specific BMIs of the participants and the other third- to sixth-grade students are provided in Table 2. No participants were underweight; 1.4% of the other third- to sixth-grade students had BMIs below the 5th percentile. No significant differences in mean age, height, weight, BMI percentiles, or the distribution within the BMI categories were found between the participants and the other third- to sixth-grade students (Table 2). More than 40% of students were categorized as at or above the 85th percentile for BMI, and ≈23% were at or above the 95th percentile. The mean (±SD)

TABLE 3
Biochemical indexes and the percentage of participants in each classification

<table>
<thead>
<tr>
<th>Value2</th>
<th>Biochemical index classification</th>
<th>Percentage of participants in each classification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total cholesterol (mg/dL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>172.3 ± 28.8</td>
<td>&lt;170</td>
</tr>
<tr>
<td></td>
<td></td>
<td>170–199</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥200</td>
</tr>
<tr>
<td></td>
<td>LDL cholesterol (mg/dL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100.0 ± 28.4</td>
<td>&lt;110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>110–130</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥130</td>
</tr>
<tr>
<td></td>
<td>HDL cholesterol (mg/dL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>56.5 ± 11.7</td>
<td>≥35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;35</td>
</tr>
<tr>
<td></td>
<td>Triacylglycerols (mg/dL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>78.5 ± 43.3</td>
<td>&lt;150</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥150</td>
</tr>
<tr>
<td></td>
<td>Fasting glucose (mg/dL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>99.8 ± 9.3</td>
<td>&lt;110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>110–125</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin A1c (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.3 ± 0.4</td>
<td>&lt;6.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥6.5</td>
</tr>
</tbody>
</table>

1 n = 90.
2 ± SD.
Prevalence of one or more risk factors for the metabolic syndrome

<table>
<thead>
<tr>
<th></th>
<th>Percentage (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥1 Risk factor</td>
</tr>
<tr>
<td>Total (n = 90)</td>
<td>35.6 (23.7, 44.0)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Boys (n = 40)</td>
<td>37.5 (22.7, 54.2)</td>
</tr>
<tr>
<td>Girls (n = 50)</td>
<td>34.0 (21.2, 48.8)</td>
</tr>
<tr>
<td>BMI status</td>
<td></td>
</tr>
<tr>
<td>Normal (n = 50)</td>
<td>26.0 (14.6, 40.3)</td>
</tr>
<tr>
<td>At risk (n = 19)</td>
<td>36.8 (16.3, 61.6)</td>
</tr>
<tr>
<td>Overweight (n = 21)</td>
<td>57.1 (34.0, 78.2)</td>
</tr>
</tbody>
</table>

1 Normal, 5th to <85th percentile; at risk, 85th to <95th percentile; overweight, ≥95th percentile.

DISCUSSION

The metabolic syndrome was included as an entity that warranted clinical intervention in the 2001 National Cholesterol Education Program’s Adult Treatment Panel III (ATP III) guidelines (12). This definition was modified by Cook et al (14) for 12–19-y-olds based on the closest representative values obtainable from the pediatric NHANES III reference data. They found that the prevalence of the metabolic syndrome was 4% overall and 28.7% among overweight adolescents. African American adolescents had the lowest rate (2.0%), followed by whites (4.8%) and Mexican Americans (5.6%). We found that the metabolic syndrome occurred in 5.6% of participants; however, it was found exclusively in those with BMIs at or above the 95th percentile (13.8%), as was abdominal obesity (14.3%) and low HDL-cholesterol concentrations (19%). Elevated BP, triacylglycerol, and glucose concentrations occurred in all BMI categories. The higher overall rates for the metabolic syndrome in our participants than in those of Cook et al probably reflects the increase in obesity prevalence that has occurred since the NHANES III data collection period (1988–1994). The higher prevalence for the metabolic syndrome among overweight 12–19-y-olds (28.7%) compared with our overweight 8–12-y-olds (13.8%) is an expected, age-related, observation. More recently, Weiss et al (28) reported that 49.7% of children (4–20 y old) with BMIs above the 97th percentile had the metabolic syndrome; among African Americans this rate dropped to 39%. Unfortunately, our sample was too small to assess rates for those with BMIs above the 97th percentile. A

Prevalence of individual risk factors for the metabolic syndrome

<table>
<thead>
<tr>
<th></th>
<th>Percentage (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Abdominal obesity</td>
</tr>
<tr>
<td>Total (n = 90)</td>
<td>6.7 (2.5, 14.0)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Boys (n = 40)</td>
<td>7.5 (1.6, 20.4)</td>
</tr>
<tr>
<td>Girls (n = 50)</td>
<td>6.0 (1.3, 16.6)</td>
</tr>
<tr>
<td>BMI status</td>
<td></td>
</tr>
<tr>
<td>Normal (n = 50)</td>
<td>0</td>
</tr>
<tr>
<td>At risk (n = 19)</td>
<td>0</td>
</tr>
<tr>
<td>Overweight (n = 21)</td>
<td>28.6 (11.3, 52.2)</td>
</tr>
</tbody>
</table>

1 Normal, 5th to <85th percentile; at risk, 85th to <95th percentile; overweight, ≥95th percentile.

WC of the participants was 64.8 ± 10.7 cm (range: 48.8–109.0 cm).

Excluding total cholesterol, participants’ mean values for lipids, glucose, and Hb A1c were within the desirable range (Table 3). When categorized by the American Heart Association guidelines, the prevalence of undesirable concentrations for total cholesterol, LDL cholesterol, and triacylglycerols ranged from 7% to 19%, and <5% of the participants’ total cholesterol concentrations were within the desirable range. Fasting glucose concentrations >125 mg/dL were not observed, and all Hb A1c concentrations were within the desirable range (<6.5 mg/dL); however, almost 15% of the participants had IFG.

The prevalence of risk factors for the metabolic syndrome is summarized in Table 4. Overall, the metabolic syndrome (presence of ≥3 risk factors) occurred in 5.6% of the participants. No significant difference was found between boys and girls (two-sided \(P = 1.0\)): 35.6% of the participants had ≥1 more risk factor, 8.9% had ≥2 risk factors, and 0% of the participants had ≥3 abnormalities. All students who met the criteria for the metabolic syndrome were overweight. No normal-weight participant had more than one risk factor. The distribution of each risk factor of the metabolic syndrome is shown in Table 5. Overall, high fasting glucose and triacylglycerol concentrations were most common, whereas abdominal obesity and low HDL cholesterol were the least common and only occurred in overweight participants. Individual risk factors were similar for boys and girls (two-sided \(P = 1.0\) for all 5 risk factors).
recent pilot study of 1700 multiethnic eighth-grade children found that 13.9% had the metabolic syndrome based on ATP III guidelines (29). These students were ≈3.5 y older than our participants, and only 23% were African American. Furthermore, plasma rather than capillary samples were used to measure lipid and glucose concentrations, and ATP III guidelines for the metabolic syndrome were used for lipid and glucose concentrations, which made direct comparisons of the results between the studies difficult. Despite the differences in design, these studies collectively document that the metabolic syndrome is present in a significant number of fairly young overweight children.

Few studies have examined capillary finger-stick screening for lipid and glucose concentrations in conjunction with anthropometric measurements in a school-based setting, particularly in low-income, urban, African American children. Muratova et al (30) reported that nonfasting finger-stick capillary samples in rural, economically disadvantaged Appalachian fifth-grade students were more sensitive than was family history in predicting elevated blood cholesterol concentrations. The same group obtained measurements for BMI and nonfasting finger-stick samples in a school-based obesity screening in (n = 1413; 98% were white) and found that mean total cholesterol was 170 mg/dL, and 25% of the students were presumptively dyslipidemic, defined as a total cholesterol concentration >200 mg/dL or an HDL-cholesterol concentration <35 mg/dL (31). Overall, 15% of their sample had systolic or diastolic hypertension (≥95th percentile), and 45% had BMIs at or above the 85th percentile. Our mean values for total cholesterol and prevalence for elevated total cholesterol and BMI are quite similar to these (mean total cholesterol: 172.3 mg/dL; 19% with elevated total cholesterol, 44% with a BMI at or above the 85th percentile, and 9% with a BP at or above the 90th percentile), despite differences in the fasted state for capillary samples, age, and ethnicity between our studies. Far fewer of our participants had low HDL-cholesterol concentrations (1.1%), as would be expected with a comparison of African American with white populations.

More than 40% of our participants had a BMI at or above the 85th percentile. Very similar rates for BMI categories were found among the other third- to sixth-grade students, which suggests that our participants were a representative sample of the target group. We are currently involved in a larger study of low-income, predominantly African American fifth- to seventh-grade students at 4 other elementary schools in the Chicago area. Among the ≈500 students we have recruited thus far, >40% have BMIs at or above the 85th percentile. A recent survey of 3000 children in kindergarten through fifth grade in New York City public schools reported that 43% of the children had BMIs at or above the 85th percentile (19% at risk of overweight, 24% overweight), results that are strikingly similar to ours (32). The Sinai Health System in Chicago recently completed a door-to-door community health survey of 1700 scientifically selected households in 6 Chicago community areas that represented the racial, ethnic, and socioeconomic diversity of the city (33). On the basis of parental reports of heights and weights for 2–12-y-old children in 3 neighborhoods with the largest African American population (ranging from 47% to 98%), >60% had BMIs at or above the 85th percentile. In the poorest of these neighborhoods, this value increased to 68%. Nationally, 15.3% of 6–11-y-olds are at or above the 95th percentile for sex- and age-specific BMI (3) and among non-Hispanic black children, this value was 19.5%. Overweight and obesity among low-income, urban, African American children are greater than the national averages. Research focused on the reasons for this is needed to effectively design and tailor interventions to meet the needs of this population.

Abdominal obesity was not found in participants with BMIs below the 95th percentile; however, it occurred in almost 30% of students with BMIs at or above this level. All of these measurements were made by a person with extensive training in anthropometrics using quality assurance methods that were tightly defined; thus, we do not believe that our WC data are plagued with systematic errors. Our students were between 8 and 12 y of age and in the midst of pubertal development, a time when body proportions are known to vary among different races. In adults, central obesity is associated with insulin resistance and individual risk factors for the metabolic syndrome (34); however, this relation is not as definitive in younger children. Weiss et al (28) used a threshold BMI z score of ≥2.0 as a risk factor rather than WC in their definition for the metabolic syndrome because of concerns over the changes that occur in body proportions and risk assessment during pubertal development. No associations between waist-to-hip ratios and traditional cardiovascular disease risk factors were reported by Valle et al (35) in 6–9 y-old children. In 8–13-y-old Hispanic children with BMIs at or above the 85th percentile and a family history of type 2 diabetes, central obesity occurred in 62% of participants (36). This is more than twice the level observed among our overweight participants; however, we did not recruit based on a positive family history of type 2 diabetes, which is known to be highly associated with central adiposity. Because the prevalence of elevated WC was lower than expected, it would have diminished rather than inflated our prevalence rates for the metabolic syndrome. Further study is needed on the role of WC on disease risk in this age group.

The cross-sectional design of this study precludes any causal inferences and limits assumptions regarding duration for all of the risk factors. The definition used for the metabolic syndrome was devised for older children; thus, our findings can only be viewed as “presumable” metabolic syndrome rates. Fasting finger-stick capillary samples were used to assess the biochemical indexes; thus, individual concentrations may be higher or lower than plasma samples. However, the manufacturer’s information indicates that these measurements are not affected by systematic error. To reduce error and overestimation of undesirable lipid and glucose concentrations, a second measurement was obtained at a later date in all children with values outside desirable ranges and the lower of the 2 values was used for analysis. Only 23% of the students in the third to sixth grades were included in our analysis for lipid abnormalities and features of the metabolic syndrome. This rate can be partially explained by the numerous independent steps required of the parents and guardians for student participation (5). Specifically, the parent or guardian had to 1) read the letter mailed home describing the study, 2) sign and return the enclosed consent form, 3) and bring their child to school ≈45 min before the beginning of the scheduled start of the school day in a fasted condition on the appointed day for finger-stick measurements. Despite this relatively low participation rate, the similarities in WC, BMI, and demographic backgrounds of our participants suggest that our findings can be generalized to the other third- to sixth-grade students in the 2 participating schools.
In conclusion, our findings confirm and expand the findings of a small but consistent number of studies that have reported excessive obesity and the metabolic syndrome in schoolchildren. These pilot data document for the first time that most of African American children (59%) attending the 2 low-income urban schools in the study were overweight, had one or more risk factors for the metabolic syndrome, or met both conditions. Additionally, 48% of the participants with BMIs at or above the 85th percentile had one or more risk factors for the metabolic syndrome. A school-based universal health screening program tailored to children in low-income minority neighborhoods may provide an efficient venue for prevention and tracking childhood obesity and its risk factors.

CLB developed the primary hypothesis and participated in the study design, data management, interpretation of results, and overall coordination of the project personnel. SG was the project coordinator, was responsible for personnel management, and participated in the subject recruitment, data collection, data entry, and manuscript preparation. HFL participated in the subject recruitment, data analysis, and interpretation of the results. KT participated in the subject recruitment, data collection, and manuscript preparation. BD was responsible for data collection, entry, cleaning, and preliminary analysis and for quality assurance. YW participated in the data analysis and was a scientific advisor. CB participated in the data analysis and manuscript preparation and was a scientific advisor. RL was a scientific advisor and assisted with study design development. There were no conflicts of interest.

REFERENCES


Effects of an ad libitum low-glycemic load diet on cardiovascular disease risk factors in obese young adults"1–3

Cara B Ebbeling, Michael M Leidig, Kelly B Sinclair, Linda G Seger-Shippee, Henry A Feldman, and David S Ludwig

ABSTRACT

Background: The optimal nutritional approach for the prevention of cardiovascular disease among obese persons remains a topic of intense controversy. Available approaches range from conventional low-fat to very-low-carbohydrate diets.

Objective: The aim of this pilot study was to evaluate the efficacy of an ad libitum low-glycemic load diet, without strict limitation on carbohydrate intake, as an alternative to a conventional low-fat diet.

Design: A randomized controlled trial compared 2 dietary treatments in obese young adults (n = 23) over 12 mo. The experimental treatment emphasized ad libitum consumption of low-glycemic-index foods, with 45–50% of energy from carbohydrates and 30–35% from fat. The conventional treatment was restricted in energy (250–500 kcal/d deficit) and fat (<30% of energy), with 55–60% of energy from carbohydrate. We compared changes in study outcomes by repeated-measures analysis of log-transformed data and expressed the results as mean percentage change.

Results: Body weight decreased significantly over a 6-mo intensive intervention in both the experimental and conventional diet groups (−8.4% and −7.8%, respectively) and remained below baseline at 12 mo (−7.8% and −6.1%, respectively). The experimental diet group showed a significantly greater mean decline in plasma triacylglycerols than did the conventional diet group (−37.2% and −19.1%, respectively; P = 0.005). Mean plasminogen activator inhibitor 1 concentrations decreased (−39.0%) in the experimental diet group but increased (33.1%) in the conventional diet group (P = 0.004). Changes in cholesterol concentrations, blood pressure, and insulin sensitivity did not differ significantly between the groups.

Conclusion: An ad libitum low-glycemic load diet may be more efficacious than a conventional, energy-restricted, low-fat diet in reducing cardiovascular disease risk. Am J Clin Nutr 2005;81:976–82.

KEY WORDS Obesity, glycemic index, glycemic load, dietary composition, weight-reducing diet, cholesterol, triacylglycerol, plasminogen activator inhibitor 1, PAI-1, young adults

INTRODUCTION

The alarming prevalence of obesity and the associated risk of cardiovascular disease (CVD) have been well documented (1) and extensively publicized in the United States. As a result, millions of obese adults are following weight-loss diets. Recently, Atkins-type very-low-carbohydrate diets have rapidly grown in popularity (2), although low-fat diets remain the cornerstone of conventional treatment based on clinical practice recommendations (3, 4). Whereas a few studies have suggested that carbohydrate-restricted diets may have significantly greater benefits than do low-fat diets in reducing CVD risk (5, 6), there is widespread concern regarding the safety and long-term efficacy of severe carbohydrate restriction (7, 8).

A low-glycemic load (GL) diet, containing unrestricted amounts of carbohydrate from low-glycemic index (GI) foods, represents an alternative to low-fat diets on the one hand and to low-carbohydrate diets on the other. The GI is defined as the incremental area under the blood glucose response curve after consumption of 50 g of available carbohydrate from a test food, divided by the area under the curve after consumption of 50 g of carbohydrate from a reference food (ie, glucose or white bread) (9). The GL is the arithmetic product of the amount of carbohydrate consumed and the GI (10) and thus describes the overall effects of both quantity and source of carbohydrate on postprandial glycemia (11). Risk of CVD has been inversely associated with dietary GI or GL in some (12–15) but not all (16) epidemiologic studies. Moreover, whereas several short-term intervention studies have described beneficial effects of low-GI diets on blood lipids in overweight adults (17–20) and on the capacity for fibrinolysis in diabetic patients (21, 22), the long-term efficacy of low-GL diets in reducing CVD risk has not previously been evaluated (23).

The aim of this pilot study was to evaluate the efficacy of an experimental ad libitum low-GL diet. We hypothesized that the experimental diet would have a more beneficial effect on CVD risk factors than would a conventional, energy-restricted, low-fat diet over a 12-mo intervention.

SUBJECTS AND METHODS

Screening and enrollment

The protocol was approved by the institutional review board at Children’s Hospital Boston, and written informed consent was obtained from each subject. Inclusion criteria included: age between 18 and 35 y, body mass index (BMI; in kg/m²) ≥27, body

1 From the Division of Endocrinology, Department of Medicine, Children’s Hospital, Boston, MA.

2 Supported by grant no. R01 DK59240 (to DSL) and grant no. K01 DK62237 (to CBE) from the National Institute of Diabetes and Digestive Kidney Diseases, the Charles H Hood Foundation (Boston, MA), and grant no. M01 RR02172 from the National Institutes of Health to support the General Clinical Research Center at Children’s Hospital Boston (Boston, MA).

3 Reprints not available. Address correspondence to DS Ludwig, Department of Medicine, Children’s Hospital, 300 Longwood Avenue, Boston, MA 02115. E-mail: david.ludwig@childrens.harvard.edu.

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weight <136 kg (300 lb), and absence of major medical illness as assessed by physical examination and laboratory screening tests (ie, kidney and liver enzymes, thyrotropin, glycosylated hemoglobin, fasting plasma glucose, and urinalysis). After being screened for eligibility, 34 obese young adults (30 females and 4 males) were enrolled in the study. Of these, 22 females and 1 male completed assessments at the end of the 12-mo intervention (Figure 1).

Study design

Subjects were randomly assigned to the experimental (low-GL diet) or conventional (low-fat diet) treatment group between August 2001 and July 2002. The study comprised a 6-mo intensive intervention (12 dietary counseling sessions) and a 6-mo follow-up (2 dietary counseling sessions). The duration of each counseling session was 1 h. Study outcomes were measured at 0, 6, and 12 mo.

Experimental diet

The experimental diet prescription was not energy restricted. Rather, we used an ad libitum approach based on previous research that suggested greater satiety and decreased voluntary energy intake among subjects consuming low-GL diets (24). Subjects were counseled to consume carbohydrate-containing foods with a relatively low GI (eg, nonstarchy vegetables, fruit, legumes, nuts, and dairy products; 24), to consume carbohydrate with protein and healthful fat at every meal and snack, and to eat to satiety and snack when hungry. A low-GL food pyramid provided the basis for nutrition education (25). The target macronutrient composition was 45–50% of energy from carbohydrate, 30–35% of energy from fat, and the remainder from protein.

Conventional diet

The conventional diet prescription was based on current recommendations for weight loss and CVD risk reduction, with emphasis on restricting energy intake by reducing dietary fat (3). Meal plans were based on an exchange system (26) designed to elicit an energy deficit of 250–500 kcal/d. Energy requirements were estimated by using the Harris-Benedict equation (27), multiplied by 1.5 to account for physical activity and adjusted for baseline dietary intake. The American Diabetes Association’s diabetes food pyramid provided the basis for nutrition education (28). The target macronutrient composition was 55–60% of energy from carbohydrate, <30% of energy from fat, and the remainder from protein.

Behavioral therapy and physical activity recommendations

Both groups received the same behavioral therapy and physical activity recommendations. Behavioral therapy focused on enhancing self-efficacy for lifestyle change by using social cognitive theory as a conceptual framework (29). Fostering behavioral capability (ie, knowledge and skill) and self-control was the primary objective during the dietary counseling sessions. Patient expectations (ie, anticipated outcomes), expectancies (ie, values
ascribed to outcomes), and perceptions of environmental influences were among the topics of discussion. To operationalize the self-control construct, the study dietitian encouraged patients to set goals around eating behaviors, to self-monitor goal attainment, and to explore solutions to problems. Physical activity recommendations were consistent with public health guidelines (30).

**Process evaluation**

The intervention process was evaluated on the basis of attendance at the dietary counseling sessions and adherence to respective diet prescriptions. All subjects received extensive instruction in keeping food diaries. Three-dimensional food models, plates, bowls, glasses, and measuring cups and spoons were used to educate subjects regarding accurate appraisal of portion sizes. The diaries were reviewed with each subject at the time of receipt to provide clarification, as necessary, on recorded foods and beverages. FOOD PROCESSOR PLUS software (version 8.2; ESHA Research, Salem, OR) was used to quantify intakes of fat, carbohydrate, protein, and fiber. The GI of individual carbohydrate-containing foods was assigned according to published values based on a glucose reference (31). Daily GL was calculated by multiplying the total amount of dietary carbohydrate in g by the weighted GI for each food and then adjusted for energy intake:

\[
\text{weighted GI} = \sum (\text{GI for food item} \\times \text{proportion of total carbohydrate contributed by item})
\]

(1)

and

\[
\text{GL} = \frac{\text{weighted GI} \times \text{grams of carbohydrate}}{1000 \text{ kcal}}
\]

(2)

To ensure that treatments were delivered according to established procedures, the study dietitian completed a tracking form and progress note immediately after each session. Seven-day food diaries were obtained at baseline (month 0), during the intensive intervention period (3 and 6 mo), and at the end of follow-up (12 mo) for evaluation of process outcomes. In addition, patients were encouraged to keep food diaries throughout the intervention as a self-monitoring strategy. Patients were not given explicit information regarding the target contributions of each macronutrient to total energy intake. Rather, the study dietitian reviewed the diaries after each counseling session and provided practical advice, as necessary, to foster eating behaviors consistent with the diet prescriptions. The project director met with the study dietitian on a regular basis to review food diaries, tracking forms, and progress notes.

**Assessment of study outcomes**

Weight and height were assessed by using an electronic scale (model 6702; Scale-Tronix, White Plains, NY) and a wall-mounted stadiometer (Holtain Limited, Crymych, United Kingdom), respectively. Body composition was measured by dual-energy X-ray absorptiometry (DXA) with the use of Hologic instrumentation (model QDR 4500; Hologic Inc, Bedford, MA). Blood pressure was determined by using an automated system (Dinamapp, Tampa, FL) while the subject sat quietly. A blood sample was drawn by venipuncture after a 12-h overnight fast.

**Laboratory analyses**

Plasma lipid concentrations were measured in a laboratory certified by the Centers for Disease Control and Prevention–National Heart, Lung, and Blood Institute Lipid Standardization Program. Total cholesterol, HDL cholesterol, and triacylglycerols were measured by using a Hitachi 911 analyzer (Roche Diagnostics, Indianapolis, IN), and LDL cholesterol was measured by using a homogeneous enzymatic assay (Genzyme Corp, Cambridge, MA) (32). Plasma concentrations of plasminogen activator inhibitor 1 (PAI-1) were measured by using an enzyme-linked immunosorbent assay (ELISA; Diagnostica Stago, Parsippany, NJ). Plasma glucose and serum insulin concentrations were measured by using a Hitachi 917 analyzer (Roche Diagnostics) and an Elecsys 2010 system (Roche Diagnostics), respectively. With the use of glucose (mg/dL) and insulin (µU/mL) concentrations, we calculated the quantitative insulin sensitivity check index: 1/(insulin + log glucose) (33).

**Statistical analysis**

We analyzed dietary data and study outcomes by repeated-measures analysis of variance. We tested each variable for change over time (0, 6, and 12 mo) and for a difference in time course between the 2 groups (experimental and conventional diet) by assessing the main effect of time and the group × time interaction, respectively. To avoid the increased risk of type I inferential error from multiple comparisons, we limited statistical testing of time trends to the overall change (from 0 to 6 to 12 mo), with the exception of 2 planned comparisons for process measures, ie, GL and dietary fat. We accounted for within-subject correlation by using a banded covariance structure, which allowed a lower correlation between the 0- and 12-mo observations than between the 0- and 6-mo or the 6- and 12-mo observations. Statistical significance was defined as \( P < 0.05 \).

The primary analysis included data from only the 23 subjects who completed the study. Secondary analyses included available data from all 34 randomly assigned subjects. Study outcomes were log transformed for analysis, and results are expressed as percentage change. Dietary data were analyzed without transformation. We used SAS software (release 9.0; SAS Institute Inc, Cary, NC) for all computations.

**RESULTS**

**Subjects**

Baseline data for the subjects who completed the study \( n = 23; 67.6\% \) of those randomly assigned to a treatment group are presented in Table 1. There were no significant differences in baseline measures between diet groups. The male who completed the study was in the conventional diet group.

**Process data**

Attendance at the 14 dietary counseling sessions approximated 100% for the 23 subjects who completed the study; 2 subjects missed just one visit each. Nutrient intake data derived from the food diaries are presented in Table 2. At baseline, we found no significant differences between groups with respect to...
The nutrients of interest. GL decreased significantly in the experimental diet group (0–6 mo, \( P < 0.001; 0–12 \text{ mo}, P < 0.001 \)) and did not change in the conventional diet group. Dietary fat decreased significantly in the conventional diet group (0–6 mo, \( P < 0.001; 0–12 \text{ mo}, P = 0.004 \)) and increased nonsignificantly in the experimental diet group.

### Outcomes

Study outcomes are presented in Table 3. Body weight decreased significantly over the 6-mo intensive intervention in the experimental and conventional diet groups, and it remained below baseline at 12 mo. Mean weight loss did not differ significantly between the groups, and there were no significant differences between the experimental and conventional diet groups in the mean percentage change in fat mass (−16.5 compared with −15.7; \( P = 0.97 \)) and lean mass (−1.1 compared with −1.5; \( P = 0.92 \)). Nevertheless, the experimental diet group showed greater mean declines in plasma triacylglycerols. Mean changes in plasma PAI-1 concentrations also differed between the groups, decreasing in the experimental diet group and increasing in the conventional diet group. Decreases in total cholesterol and increases in HDL cholesterol were marginally nonsignificant and did not differ significantly between groups. There were no significant changes in LDL cholesterol or blood pressure in either group throughout the study. The insulin sensitivity index increased significantly in both groups. Results were materially unchanged in the secondary analyses that included data from all randomly assigned subjects (data not presented).

### DISCUSSION

In light of widespread concern regarding the high toll of the obesity epidemic on human suffering (34) and health care costs (35), development of effective weight-management strategies is a public health priority (36). Debate about the appropriate diets for promoting weight loss and decreasing CVD risk has focused largely on the metabolic effect of dietary carbohydrate and fat (37–41). Obesity has become increasingly prevalent over the last 2 decades (42), and the contribution of carbohydrate to total energy intake has increased in tandem with a reduction in the contribution of fat (43). The increase in carbohydrate intake can be largely attributed to consumption of high-GI foods (44). Taken together, these observations suggest that both the quantity and the source of carbohydrate may be important considerations.

To our knowledge, the randomized controlled trial presented herein is the first long-term study comparing a low-GL diet, with emphasis on consumption of low-GI sources of carbohydrate, with a low-fat diet for decreasing CVD risk in obese young adults. We hypothesized that less hunger or greater satiety in response to an ad libitum low-GL diet may facilitate a decrease in energy intake (45), without the need for externally imposed energy restriction. Our hypothesis is supported, in that mean weight loss among persons following the ad libitum low-GL diet and mean weight loss among persons following the energy-restricted low-fat diet did not differ significantly during the intensive 6-mo intervention (−8.4% and −7.8%, respectively), and there was no significant weight rebound during the follow-up. Moreover, we previously observed greater decreases in BMI and fat mass among adolescents in response to a low-GL diet than in response to a low-fat diet (46). Differences in the response to an energy-restricted diet between adolescents and adults, particularly women, may partially explain the varied patterns of weight loss between our 2 studies. Adolescents have a strong desire for autonomy and seem to resist the use of an exchange system that imposes energy restriction; for this reason, the flexibility of an ad libitum approach may be especially beneficial in this age group. In contrast, many young women are accustomed to following conventional energy-restricted diets, which may limit the likelihood of seeing a group effect over a 12-mo period in this patient population. Additional studies are needed to examine group effects with longer-term follow-up. Nevertheless, our findings compare favorably with those of studies evaluating the effect of severe carbohydrate restriction on weight loss (5, 6). Foster et al (5) observed decreases in body weight of 4.4% and 2.5% at 12 mo among patients prescribed carbohydrate-restricted and low-fat diets, respectively. In a similar 12-mo study, Stern et al (6) observed decreases of 3.5% and 2.4%.

A low-GL diet, such as that used in the present study, may represent an optimal compromise between low-fat diets at one end of the spectrum and carbohydrate-restricted diets at the other. Although changes in body weight did not differ between the 2 groups, the metabolic benefits of a low-GL diet in decreasing CVD risk may be significantly greater than those achieved with either of the more restrictive approaches. The low-fat diet had a significantly less favorable effect on circulating triacylglycerol and PAI-1 concentrations than did the low-GL diet. Indeed, low-fat diets typically have a high carbohydrate content, which causes postprandial hyperglycemia and hyperinsulinemia (47). In turn, these episodes may enhance hepatic triacylglycerol production or reduce peripheral clearance (39, 48) and also promote the synthesis and secretion of PAI-1 (49) via plausible physiologic and molecular mechanisms. Attention has been directed toward controlling triacylglycerol and PAI-1 concentrations in light of the direct associations between these variables and cardiovascular events (50, 51). Whereas very low-carbohydrate diets have beneficial effects on triacylglycerol concentrations (perhaps as a result of their low GL), the sustainability of such highly restrictive diets over the long term is questionable (5, 6). A low-GL diet,
containing moderate amounts of carbohydrate and fat, offers a potentially more flexible approach. In contrast to very-low-carbohydrate diets, the reduction in GL in the experimental group was achieved by a relatively small decrease in carbohydrate intake that was accompanied by a substantial reduction in GI. Nevertheless, the mean decrease in triacylglycerol concentration in the experimental group over 12 mo (37.2%) compares favorably with decreases of 17.0% (5) and 28.6% (6) in previous studies of very-low-carbohydrate diets. Data from metabolic studies, epidemiologic investigations, and clinical trials lend support to the efficacy of eating patterns that are consistent with a low-GL diet (38), including consumption of vegetables, fruit, and whole grains as primary sources of carbohydrate. Moreover, our findings extend data from previous short-term studies showing beneficial reductions in triacylglycerol concentrations with low-GI diets (52–55).

Several issues pertaining to study design warrant consideration. Strengths of the study include the use of treatments of equal intensity in both experimental and control diet groups, which would eliminate this factor as a source of confounding; excellent attendance at counseling sessions among those who completed the study; a longer follow-up than in previous studies of GL or GI and CVD risk factors (17, 18, 20–22); and careful attention to process evaluation. Moreover, changes in dietary fiber, a frequently cited confounder in evaluations of GI or GL (56, 57), were similar between groups. Limitations include the self-reporting of dietary intakes for process evaluation, reliance on published GI values for calculating dietary GL (31), and a small, predominately female sample from which there was some attrition. Underreporting of dietary intake is a well-recognized phenomenon in all outpatient studies aiming to assess the effects of

### Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Experimental diet (n = 11)</th>
<th>Conventional diet (n = 12)</th>
<th>Group</th>
<th>Time</th>
<th>Group × time interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycemic load (g/1000 kcal)</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Baseline</td>
<td>77.2 ± 5.7</td>
<td>77.8 ± 2.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interim</td>
<td>54.4 ± 2.0</td>
<td>78.4 ± 1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 mo</td>
<td>53.0 ± 2.7</td>
<td>77.1 ± 2.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycemic index</td>
<td></td>
<td></td>
<td>0.006</td>
<td>&lt;0.001</td>
<td>0.004</td>
</tr>
<tr>
<td>Baseline</td>
<td>56.2 ± 1.2</td>
<td>56.6 ± 1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interim</td>
<td>46.2 ± 1.6</td>
<td>52.8 ± 0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 mo</td>
<td>46.3 ± 2.0</td>
<td>52.9 ± 1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrate (% of energy)</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td>0.43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Baseline</td>
<td>52.7 ± 2.2</td>
<td>54.8 ± 1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interim</td>
<td>47.2 ± 1.6</td>
<td>59.4 ± 0.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 mo</td>
<td>45.5 ± 1.1</td>
<td>58.3 ± 1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total fat (% of energy)</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td>0.03</td>
<td>0.006</td>
</tr>
<tr>
<td>Baseline</td>
<td>32.6 ± 1.6</td>
<td>30.0 ± 1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interim</td>
<td>33.0 ± 1.2</td>
<td>23.4 ± 0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 mo</td>
<td>35.4 ± 1.2</td>
<td>24.3 ± 2.0</td>
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<td></td>
</tr>
<tr>
<td>Saturated fat (% of energy)</td>
<td></td>
<td></td>
<td>0.04</td>
<td>&lt;0.001</td>
<td>0.18</td>
</tr>
<tr>
<td>Baseline</td>
<td>11.3 ± 0.8</td>
<td>10.7 ± 0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interim</td>
<td>9.1 ± 0.7</td>
<td>7.5 ± 0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 mo</td>
<td>10.6 ± 0.9</td>
<td>7.6 ± 0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td></td>
<td></td>
<td>0.17</td>
<td>&lt;0.001</td>
<td>0.07</td>
</tr>
<tr>
<td>Baseline</td>
<td>15.7 ± 1.0</td>
<td>16.1 ± 0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interim</td>
<td>21.1 ± 1.1</td>
<td>18.7 ± 0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 mo</td>
<td>20.5 ± 0.9</td>
<td>18.1 ± 1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fiber (g/1000 kcal)</td>
<td></td>
<td></td>
<td>0.22</td>
<td>&lt;0.001</td>
<td>0.53</td>
</tr>
<tr>
<td>Baseline</td>
<td>9.6 ± 1.0</td>
<td>8.2 ± 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interim</td>
<td>14.9 ± 1.3</td>
<td>12.6 ± 1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 mo</td>
<td>13.5 ± 1.1</td>
<td>12.8 ± 1.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td></td>
<td></td>
<td>0.84</td>
<td>&lt;0.001</td>
<td>0.76</td>
</tr>
<tr>
<td>Baseline</td>
<td>1860 ± 72</td>
<td>1802 ± 116</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interim</td>
<td>1391 ± 79</td>
<td>1409 ± 46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 mo</td>
<td>1494 ± 82</td>
<td>1472 ± 85</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Testing for overall difference in level between experimental and conventional diet groups (main effect of group), change over time (main effect of time), and difference in time course between groups (group × time interaction). Repeated-measures ANOVA was used to account for within-subject correlations.

2 ± SEM (all such values).

3 Testing for equal means in experimental and conventional diet groups by independent-sample t test found no significant baseline differences for any of the listed nutrients.

4 Means of data collected at 3 and 6 mo.
The study provides a rationale for conducting long-term, larger-scale term dietary intervention studies (5, 6).

In conclusion, a low-GL diet containing moderate amounts of carbohydrate from low-GI sources may be more efficacious than a conventional low-fat diet in reducing CVD risk. The greater benefits in response to an ad libitum diet, compared with an energy-restricted diet, are particularly noteworthy. This pilot study provides a rationale for conducting long-term, larger-scale studies comparing the effects of low-GL, low-fat, and very-low-carbohydrate diets on CVD risk among obese persons.

We thank Gary Bradwin for analysis of plasma lipids and plasminogen activator inhibitor 1, Catherine Murphy for help with data collection, and Irena Clark and Erica Garcia-Lago for technical assistance.

All authors contributed to the interpretation of results. CBE and DSL designed the study, provided oversight, and wrote the manuscript. MML was responsible for dietary counseling. KBS and LGS conducted the process evaluations to assess adherence to diet prescriptions. HAF provided consultation on statistical analysis of the data. None of the authors had any personal or financial conflict of interest.

**REFERENCES**


Blood pressure change with weight loss is affected by diet type in men1–3

Caryl A Nowson, Anthony Worsley, Claire Margerison, Michelle K Jorna, Sandra J Godfrey, and Alison Booth

ABSTRACT
Background: Weight loss reduces blood pressure, and the Dietary Approaches to Stop Hypertension (DASH) diet has also been shown to lower blood pressure.

Objective: Our goal was to assess the effect on blood pressure of 2 weight-reduction diets: a low-fat diet (LF diet) and a moderate-sodium, high-potassium, high-calcium, low-fat DASH diet (WELL diet).

Design: After baseline measurements, 63 men were randomly assigned to either the WELL or the LF diet for 12 wk, and both diet groups undertook 0.5 h of moderate physical activity on most days of the week.

Results: Fifty-four men completed the study. Their mean (±SD) age was 47.9 ± 9.3 y (WELL diet, n = 27; LF diet, n = 27), and their mean baseline home systolic and diastolic blood pressures were 129.4 ± 11.3 and 80.6 ± 8.6 mm Hg, respectively. Body weight decreased by 4.9 ± 0.6 kg (±SEM) in the WELL group and by 4.6 ± 0.6 kg in the LF group (P < 0.001 for both). There was a greater decrease in blood pressure in the WELL group than in the LF group (between-group difference (week 12 – baseline) in both SBP (5.5 ± 1.9 mm Hg; P = 0.006) and DBP (4.4 ± 1.2 mm Hg; P = 0.001]).

Conclusions: For a comparable 5-kg weight loss, a diet high in low-fat dairy products, vegetables, and fruit (the WELL diet) resulted in a greater decrease in blood pressure than did the LF diet. This dietary approach to achieving weight reduction may confer an additional benefit in reducing blood pressure in those who are overweight.

KEY WORDS Weight loss, blood pressure, diet, potassium, calcium

INTRODUCTION
Hypertension is an important public health issue and contributes to the incidence of stroke and coronary heart disease (1). The prevalence of hypertension in Australia was recently shown to be ≈29% (2). Furthermore, hypertension accounts for 6.1% of the total problems managed in general practice (3). Education pertaining to nutrition and weight accounted for 10% of all non-pharmacologic treatments provided by general practitioners and was one of the 3 most common forms of advice (3). Around the world, the incidence of overweight and obesity has increased (4). The prevalence of obesity in Australia has more than doubled in the past 20 y, and almost 60% of adults have been estimated to be overweight or obese (5). There is a direct positive relation between overweight and hypertension, such that it has been estimated that the control of obesity may eliminate 48% of the hypertension in whites (6). Dietary sodium increases blood pressure (BP), whereas dietary potassium lowers the risk of hypertension and stroke. In a controlled intervention study, a multifaceted dietary approach (DASH: Dietary Approaches to Stop Hypertension) that included a diet high in fruit, vegetables, and low-fat dairy products was shown to result in large decreases in BP (11 mm Hg systolic and 5 mm Hg diastolic pressure in hypertensive persons and 5 mm Hg systolic and 3 mm Hg diastolic in normotensive persons) (7). Therefore, the aim of the present study was to determine the effect on BP of a DASH-type weight-loss diet (WELL diet) and to compare this with usual low-fat dietary advice (LF diet) in free-living individuals who selected and prepared their own food.

SUBJECTS AND METHODS

Subjects
Subjects were recruited through newspaper articles advertising the study and at BP measurement sessions provided in workplaces and at the study center. Subjects were eligible if they were male, aged > 25 y, and had a seated office BP of ≥120 mm Hg systolic blood pressure (SBP) or ≥80 mm Hg diastolic blood pressure (DBP) at their first visit (mean of the last 3 of 4 measurements taken at 1-min intervals). Subjects who were taking antihypertensive medication were included, provided they were willing to maintain their current medication level. Subjects were excluded if they had experienced a cardiovascular event in the past 6 mo, had insulin-dependent diabetes, were taking medications such as warfarin or phenytoin, ate their main meal outside the home more than twice per week, drank >30 standard (10 g alcohol) alcoholic drinks per week, were planning to change smoking habits, or were unwilling to cease taking dietary supplements (including vitamins). Subjects were included if they had a body mass index (BMI; in kg/m2) between 25 and 35. All subjects provided written informed consent before starting the study, which was approved by the Deakin University Human Research Ethics Committee.

1 From the Centre for Physical Activity and Nutrition, School of Exercise and Nutrition Sciences, Deakin University, Burwood Highway, Burwood, Australia.
2 Supported by the Dairy Research and Development Corporation.
3 Address reprint requests to CA Nowson, School of Exercise and Nutrition Sciences, Deakin University, 221 Burwood Highway, Burwood, VIC 3125 Australia. E-mail: nowson@deakin.edu.au.
4 Received June 29, 2004.
5 Accepted for publication December 14, 2004.
Two hundred twenty persons responded to advertisements, and 165 of these were sent a screening questionnaire and invited to attend further screening. Ninety-four men attended one screening appointment, and 63 who met the entry criteria and wished to participate undertook baseline home BP measurements for 2 wk and were then randomly assigned to either the LF or the WELL diet.

Study design

Subjects were seen twice at baseline, and commenced a 12-wk intervention study and were seen at weeks 2, 4, 8, and 12. Phone contact was made with the subjects at weeks 6 and 10. Clinical BP, height, and weight were measured at baseline. Subjects monitored their home BP daily for 2 wk before being randomly assigned (stratified by antihypertensive medication use) to 1 of the 2 diets. Randomization was performed by the chief investigator (CAN) with the use of a random number generator in blocks of 8 (EXCEL 2000; Microsoft Corporation, Redmond, WA).

Anthropometry and blood pressure measurement

Height was measured with a wall-mounted stadiometer. Body weight was measured at each visit on a digital scale while the subjects wore light clothing and no shoes. Waist circumference was measured with a fiberglas tape measure anteriorly halfway between the lowest lateral portion of the ribcage and the iliac crest. Home BP was measured on the left arm with the use of an automated BP monitor (AND UA-767-PC; A&D Co Ltd, Tokyo, Japan). Subjects were trained to correctly apply the cuff and were instructed to take their BP measurements alone, at the same time of day, after 5 min of rest in a quiet room and to take 3 measurements with 1 min in between (the mean of the last 2 measurements taken each day was used in the analysis). BP measurement data were downloaded directly to a computer by the study staff at the end of each fortnight.

Biochemical indexes

Fasting (10 h overnight) blood samples were collected at baseline and at the end of the study. Serum total cholesterol, HDL cholesterol, and triacylglycerol were measured on the Hitachi 704 analyzer by using enzymatic reagents (Boehringer, Mannheim, Germany). LDL cholesterol was calculated by use of the Friedewald equation (8).

Dietary assessment

Subjects completed a 24-h dietary record each fortnight on the day before their visit with study staff. Trained research personnel checked this record. Dietary information was entered into a dietary analysis program (FOODWORKS, Professional Edition, version 3.02; Xyris Software, New York, NY) to calculate daily nutrient intakes. The mean of two 24-h records at baseline and the mean of four 24-h records at weeks 2, 4, 8, and 12 of the intervention were used in the analysis. A food-frequency questionnaire was completed at baseline and at week 12 to assess usual intake of fruit, vegetables, and dairy products.

Lifestyle intervention

Subjects were assisted with setting goals for exercise and diet. At each visit, a trained dietitian set dietary and exercise goals (≥3 goals per visit, including ≥1 for exercise and ≥1 for diet). Recipes, educational materials (diet and exercise), and tips to encourage compliance were provided to all subjects.

Dietary instruction

Dietary counseling was overseen by the coordinating dietitian (CM) and was provided by trained research staff. The WELL diet was based on our previous OZDASH diet (9), which had been modified from the US DASH diet (7). This diet included advice to consume ≥4 servings of fruit or fruit juice [1 serving = 1 medium piece of fruit (100 g) or fruit juice (200 mL)], ≥4 servings of vegetables [1 serving = 0.5 cup cooked vegetables (50 g), 1 cup salad vegetables, or 1 medium potato] and ≥3 servings of nonfat dairy products [1 serving = milk (200 mL), yogurt (200 g), or cottage or ricotta cheese (0.5 cup)] per day. Fish (1 serving = 120 g cooked) was to be consumed ≥3 times per week, legumes (1 serving = 1 cup cooked) at least once per week, and unsalted nuts and seeds (1 serving = 30 g) 4 times per week. Red meat was restricted to no more than 2 servings (1 serving = 90–100 g cooked) per week and fat to a maximum of 4 servings (4 teaspoons) per day. Subjects were advised to avoid butter, added salt (table or cooking), and obviously salty foods and to use lower-salt (<380 mg Na per 100 g) mono- or polyunsaturated margarine. Those in the WELL group received a detailed dietary information booklet, recipes, and simple advice (tips).

The LF group was advised to limit their intake of high-energy foods and drinks, reduce their saturated fat intake, choose mainly plant-based foods, consume nonfat or reduced-fat milk and yogurt, limit their cheese and ice cream intake to twice per week, select lean meat, and avoid frying foods in fat. No specific targets were set. The “Healthy Weight Guide” booklet by the National Heart Foundation of Australia (2002) was provided, together with the same recipes and tips as received by the WELL group. A maximum of 4 caffeine-containing drinks per day (eg, cola drinks, coffee, and tea) and 4 standard (10-g alcohol) alcoholic drinks per week were permitted for both diet groups.

Several incentives were included. Both groups received measuring cups and spoons and individual feedback on their daily intake of fruit and vegetables. Those in the WELL group also received individual feedback compared with the specified diet targets (fruit, vegetables, and dairy). A dairy product of their choice [eg, 200-mL tub of nonfat yogurt or a packet of reduced-fat (<15% fat) cheese slices] was offered once during the study to all subjects. Subjects could also participate in a drawing to win a double movie pass, and feedback on the group’s progression regarding targets and weight loss was provided graphically throughout the study.

The main difference between the LF diet and the WELL diet was that the WELL diet had specified targets for fruit, vegetable, and dairy intake, whereas the LF diet provided general guidelines focusing on increasing fruit and vegetable intake and reducing fat intake, particularly saturated fat.

Physical activity

All subjects were required to participate in moderate-intensity exercise for ≥30 min on all or most days of the week. The “Be Active Every Day” booklet by the National Heart Foundation (1999) was given to each participant, and individual exercise goals were set at each visit. Information was provided on calculating maximum heart rate [220 − age (y)], and subjects were advised to increase their heart rate to 60–79% of their maximum heart rate to reach a moderate level of exercise intensity and to maintain this for ≥30 min for each session. The amount of walking was monitored by using the CHAMPS questionnaire.
(10) at baseline (for 4 wk) and the average hours per week calculated for the intervention period.

**Statistical analysis**

Data were analyzed by using SPSS for WINDOWS (version 11.0; SPSS Inc, Chicago, IL) to calculate the descriptive statistics and perform the regression analysis. Mean home BP readings were calculated for each 2-wk period. Unpaired Student’s *t* tests were used to evaluate the difference between the LF and WELL diets in the changes between baseline and the last visit. *P* values of 0.05 were considered to be significant. Additionally, the effect of the diet intervention was assessed by using two-factor repeated-measures analysis of variance (diet × time) with covariates of baseline weight and BP included in specific analysis where indicated. Baseline values were used as covariates in GLM univariate analysis of variance to calculate the adjusted mean changes to test for the difference between groups for the dietary data only.

**RESULTS**

Nine subjects dropped out before completing the study (4 in the LF group and 5 in the WELL group); the subjects who dropped out did not differ significantly from the rest of the group with respect to age or BMI. Eight found it too difficult to comply with the study demands, and one moved interstate.

Of the 54 men who completed the study, 18 were taking antihypertensive medications (9 WELL, 9 LF). Subjects in the WELL group who were receiving antihypertensive therapy included 4 receiving single therapy [1 taking an angiotensin-converting enzyme (ACE) inhibitor, 1 taking a calcium-channel blocker, and 2 taking angiotensin II receptor antagonists (AT1)] and 5 receiving combination therapy (AT1 + β-blocker, n = 1; AT1 + diuretic, n = 2; AT1 + calcium-channel blocker, n = 1; ACE inhibitor + diuretic, n = 1). Subjects in the LF group who were receiving antihypertensive therapy included 5 receiving single therapy (2 taking an ACE inhibitor, 1 taking an AT1, and 2 taking calcium-channel blockers) and 4 receiving combination therapy (ACE inhibitor + diuretic, n = 1; AT1 + diuretic, n = 1; ACE inhibitor + diuretic, n = 1; AT1 + diuretic + β-blocker, n = 1).

At baseline, there were no significant differences in dietary intakes of fruit, vegetables, or calcium-containing dairy products between groups; however, those randomly assigned to the LF group were heavier, were taller, and had a greater waist measurement and had a BMI of 31 compared with 30 for the WELL group (Table 1 and Table 2).

**Effects of the intervention**

The amount of time spent walking increased in both groups over the intervention period, with no significant difference between the groups: WELL group increased to 4.4 ± 0.7 (±SEM) h/wk and LF group to 4.6 ± 0.5 h/wk (both *P* < 0.01 for the change from baseline).

There were no significant differences between the groups at baseline in fruit, vegetable, and dairy intakes as recorded on the food-frequency questionnaire. At week 12, the WELL group reported a higher intake of dairy products, but there was no significant difference between the groups in fruit and vegetable intakes. Fruit intake increased significantly during the diet compared with baseline for both groups (both *P* = 0.001; Table 3).

For the WELL group only, intakes of dairy products and vegetables were significantly higher during the diet than at baseline (dairy *P* = 0.001, vegetables *P* = 0.001; Table 3).

After adjustment for baseline dietary intake, the 24-h dietary records indicated that the reductions in dietary fat (g/d), saturated fat (g/d), percent of energy from fat, percent of energy from saturated fat, and sodium (mg/d) were greater in the WELL group than in the LF group, and the increases in the percent of energy from protein, percent of energy from carbohydrate, potassium (mg/d), calcium (mg/d), magnesium (mg/d), and phosphorus (mg/d) were greater in the WELL group than in the LF group (Table 3).

**Weight and blood pressure changes**

Weight decreased significantly in both groups by ≈5.0 kg (*P* < 0.001 for both), with subjects in the WELL group losing 6% of body weight (*P* < 0.001) and those in the LF group losing 5% (*P* < 0.001; Table 2). The rate of weight loss was not significantly different between the diet groups throughout the study. In the first 2 wk, weight loss was 1.2 ± 0.2 kg in the WELL group and 1.5 ± 0.2 kg in the LF group, and the effect did not differ significantly between diet groups (two-factor ANOVA: time × diet effect, NS).

The greatest decrease in BP in both groups was seen after 4 wk of intervention (Figure 1). There was a greater decrease in the WELL group than in the LF group [between-group difference (week 12 − baseline) in both SBP (5.5 ± 1.9 mm Hg; *P* = 0.006) and DBP (4.4 ± 1.2 mm Hg; *P* = 0.001)], Pulse rate also fell by 3.8 ± 1.6 beats/min more in the WELL group (*P* = 0.023; Table 2).

The percentage decrease in SBP was 5.5 ± 1.0% in the WELL group compared with 1.4 ± 0.9% in the LF group. The percentage decrease in DBP was 6.4 ± 1.1% in the WELL group compared with 1.0 ± 1.0% in the LF group. The significance of the difference in the percentage change between groups was *P* = 0.005 (SBP) and *P* = 0.001 (DBP).

After adjustment for baseline BP and body weight, the difference in the decrease in SBP and DBP between groups remained [SBP: 5.2 ± 1.8 mm Hg (*P* = 0.006); DBP: 4.8 ± 1.3 mm Hg (*P* = 0.001)]. Overall, there was a significant effect of diet on BP (*SBP, *P* = 0.006; DBP, *P* = 0.001; *n* = 54; repeated measures, two-factor ANOVA: time × diet effect). Adding baseline body

---

**Table 1**

Baseline characteristics of the study groups

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>WELL diet group</th>
<th>LF diet group</th>
</tr>
</thead>
<tbody>
<tr>
<td>47.1 ± 10.3</td>
<td>48.8 ± 8.3</td>
<td></td>
</tr>
<tr>
<td>Age range (y)</td>
<td>30–66</td>
<td>26–64</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>172.5 ± 5.2</td>
<td>174.4 ± 5.3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.6 ± 2.3</td>
<td>31.2 ± 2.4</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>108.2 ± 7.7</td>
<td>110.3 ± 6.7</td>
</tr>
<tr>
<td>Office SBP (mm Hg)</td>
<td>136.4 ± 16.2</td>
<td>133.6 ± 9.7</td>
</tr>
<tr>
<td>Office DBP (mm Hg)</td>
<td>88.0 ± 9.7</td>
<td>88.7 ± 6.0</td>
</tr>
<tr>
<td>Office pulse (beats/min)</td>
<td>69.3 ± 12.3</td>
<td>73.2 ± 9.9</td>
</tr>
</tbody>
</table>

1 WELL, DASH-type weight-loss diet (moderate sodium, high potassium, high calcium, low fat, with less red meat and more fish); LF, low-fat diet; SBP, systolic blood pressure; DBP, diastolic blood pressure.

2 ± SD (all such values).

3 Significantly different from the WELL diet group (unpaired *t* test): *P* < 0.01, *P* < 0.05, *P* < 0.001.
weight as a covariate to the model did not significantly affect the results (SBP, $P = 0.001$; DBP, $P = 0.001$; $n = 54$; repeated-measures, two-factor ANOVA: time × diet effect). Additionally, a model that included baseline BP and body weight as covariates did not significantly affect the results (SBP, $P = 0.003$; DBP, $P = 0.001$; $n = 54$; repeated-measures, two-factor ANOVA: time × diet effect). The pulse rate was lower in the WELL group than in the LF group ($P = 0.031$; $n = 54$; repeated-measures, two-factor ANOVA: time × diet effect).

Serum total cholesterol, HDL cholesterol, and LDL cholesterol decreased significantly in both groups by the end of the study; however, there was no significant change in triacylglycerol in the LF group and a tendency for a decrease in the WELL group of $0.3 ± 0.1$ mmol/L ($P = 0.051$; Table 2).

Regression analysis indicated that initial weight was not related to changes in SBP and DBP. However, the percentage weight loss was related to the percentage change in SBP and DBP (SBP: $R^2 = 0.16$, $\beta$ (±SE) = $0.65 ± 0.20$, $P = 0.003$; DBP: $R^2 = 0.16$, $\beta$ = $0.73 ± 0.23$, $P = 0.003$); a 10% change in weight was associated with a 7% decrease in both SBP and DBP. Univariate linear regression analysis indicated that the increase in total dairy product intake was associated with the decrease in DBP ($R^2 = 0.118$, $\beta = 0.959 ± 0.364$, $P = 0.011$) and the increase in vegetable intake was associated with the decrease in DBP ($R^2 = 0.071$, $\beta = 0.968 ± 0.487$, $P = 0.052$).

**DISCUSSION**

The present study investigated the effects on home BP of 2 dietary interventions—one based on the DASH dietary pattern and the other a usual low-fat diet—combined with increased physical activity to achieve weight loss. We found that the subjects in both diet groups achieved a weight loss of $\approx 5$–6% of body weight over 3 mo. Those in the WELL group, however, had greater decreases in SBP and DBP of $\approx 5$ and 4 mm Hg, respectively. The groups were well matched at baseline for BP and for the number of subjects taking antihypertensive medication (33% in each group), although BMI was initially one unit higher in the LF group than in the WELL group. This difference, however, is unlikely to have contributed to the increased effectiveness of the WELL diet with respect to BP, because there was no significant difference in percentage weight loss between the groups.

The reason for the greater decrease in BP with the WELL diet is not clear. We found no significant difference between the 2 groups in the change in blood lipids, although those in the WELL diet group did appear to have a greater reduction in total fat and particularly saturated fat intake. Our power to detect a difference in blood lipids, however, was low because of the limited number of subjects.

Some of the dietary differences between the WELL and the LF diet may explain some of the improved BP-lowering effect of the WELL diet, specifically, the increase in dietary potassium, which has been shown to lower BP by $\approx 3$ mm Hg systolic and 2 mm Hg diastolic (11). Dietary calcium and magnesium have also been weakly associated with lower BP in population studies (12, 13), although evidence for a BP-lowering effect in controlled intervention studies is not consistent (14). It appears, however, that a diet combining these nutrient changes—eg, lower sodium and saturated fat and higher potassium, calcium, magnesium, and phosphorus—within a diet and physical activity pattern that induces negative energy balance achieves a greater reduction in BP than does a low-fat diet.

The food-frequency questionnaire indicated a difference between groups at the end of the study in dairy intake only and not in fruit and vegetable intakes. This likely reflects the insensitivity of a food-frequency questionnaire in picking up relatively small changes in food intake. It may also indicate that the increase in dairy products, when combined with more vegetables, is a significant factor with respect to BP reduction, particularly because the linear regression analysis indicated that the change in total dairy intake together with the change in vegetable intake was univariately associated with the reduction in DBP.
### Table 3

Intervention nutrient outcomes at baseline and 12 wk by randomized group assignment

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>WELL diet group (n = 27)</th>
<th>LF diet group (n = 27)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fiber (g/d)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.2 ± 0.2</td>
<td>2.7 ± 0.3</td>
<td>0.147</td>
</tr>
<tr>
<td>Intervention</td>
<td>3.8 ± 0.2</td>
<td>3.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>1.5 (1.0, 1.9)</td>
<td>1.1 (0.6, 1.5)</td>
<td>0.210</td>
</tr>
<tr>
<td><strong>Fruit (servings/d)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.2 ± 0.2</td>
<td>2.6 ± 0.2</td>
<td>0.262</td>
</tr>
<tr>
<td>Intervention</td>
<td>3.4 ± 0.2</td>
<td>3.0 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>1.1 (0.6, 1.6)</td>
<td>0.5 (0.0, 0.9)</td>
<td>0.075</td>
</tr>
<tr>
<td><strong>Protein (% of energy)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>4.0 ± 0.2</td>
<td>2.9 ± 0.3</td>
<td>0.147</td>
</tr>
<tr>
<td>Intervention</td>
<td>4.0 ± 0.2</td>
<td>2.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>1.5 (1.0, 2.0)</td>
<td>−0.0 (−0.5, 0.5)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Energy (MJ/d)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>10.4 ± 0.6</td>
<td>11.3 ± 0.5</td>
<td>0.262</td>
</tr>
<tr>
<td>Intervention</td>
<td>8.4 ± 0.3</td>
<td>8.7 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>−2.4 (−3.0, −1.7)</td>
<td>−2.3 (−2.9, −1.6)</td>
<td>0.810</td>
</tr>
<tr>
<td><strong>Fat (g/d)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>85.8 ± 6.9</td>
<td>101.2 ± 8.3</td>
<td>0.160</td>
</tr>
<tr>
<td>Intervention</td>
<td>44.0 ± 3.3</td>
<td>57.4 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>−48.4 (−56.0, −40.8)</td>
<td>−37.2 (−44.8, −29.6)</td>
<td>0.042</td>
</tr>
<tr>
<td><strong>Saturated fat (g/d)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>31.5 ± 3.2</td>
<td>38.7 ± 3.2</td>
<td>0.122</td>
</tr>
<tr>
<td>Intervention</td>
<td>11.5 ± 1.0</td>
<td>19.2 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>−23.3 (−25.8, −20.7)</td>
<td>−16.1 (−18.7, −13.6)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Carbohydrate (g/d)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>302.4 ± 19.8</td>
<td>307.6 ± 13.1</td>
<td>0.827</td>
</tr>
<tr>
<td>Intervention</td>
<td>289.8 ± 11.4</td>
<td>270.1 ± 13.0</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>−14.6 (−38.2, 9.0)</td>
<td>−35.5 (−59.1, −12.0)</td>
<td>0.214</td>
</tr>
<tr>
<td><strong>Cholesterol (mg/d)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>323.7 ± 31.1</td>
<td>331.7 ± 24.6</td>
<td>0.840</td>
</tr>
<tr>
<td>Intervention</td>
<td>184.3 ± 16.0</td>
<td>237.1 ± 21.0</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>−143.5 (−181.4, −105.6)</td>
<td>−90.5 (−128.4, −52.7)</td>
<td>0.052</td>
</tr>
<tr>
<td><strong>Protein (% of energy)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>16.8 ± 0.6</td>
<td>6.6 ± 0.7</td>
<td>0.814</td>
</tr>
<tr>
<td>Intervention</td>
<td>19.8 ± 0.5</td>
<td>19.5 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>3.0 (1.9, 4.1)</td>
<td>2.8 (1.7, 3.9)</td>
<td>0.047</td>
</tr>
<tr>
<td><strong>Fat (% of energy)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>29.9 ± 1.4</td>
<td>31.8 ± 1.6</td>
<td>0.380</td>
</tr>
<tr>
<td>Intervention</td>
<td>18.5 ± 1.1</td>
<td>23.8 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>−12.0 (−14.3, −9.8)</td>
<td>−7.3 (−9.6, −5.1)</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>Saturated fat (% of energy)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>10.8 ± 0.7</td>
<td>12.1 ± 0.7</td>
<td>0.184</td>
</tr>
<tr>
<td>Intervention</td>
<td>4.9 ± 0.4</td>
<td>8.0 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>−6.5 (−7.3, −5.7)</td>
<td>−3.6 (−4.5, −2.8)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Carbohydrate (% of energy)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>49.4 ± 1.7</td>
<td>47.3 ± 1.8</td>
<td>0.396</td>
</tr>
<tr>
<td>Intervention</td>
<td>59.1 ± 1.4</td>
<td>53.5 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>10.4 (7.6, 13.3)</td>
<td>5.5 (2.6, 8.3)</td>
<td>0.017</td>
</tr>
<tr>
<td><strong>Alcohol (g/d)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>12.0 ± 3.3</td>
<td>17.6 ± 4.5</td>
<td>0.325</td>
</tr>
<tr>
<td>Intervention</td>
<td>6.4 ± 2.5</td>
<td>10.1 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>−6.8 (−11.5, −2.2)</td>
<td>−6.2 (−10.9, −1.6)</td>
<td>0.862</td>
</tr>
<tr>
<td><strong>Alcohol (% of energy)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.5 ± 1.0</td>
<td>4.1 ± 1.0</td>
<td>0.631</td>
</tr>
<tr>
<td>Intervention</td>
<td>3.1 ± 1.0</td>
<td>4.2 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>−0.5 (−2.1, 1.2)</td>
<td>0.2 (−1.4, 1.9)</td>
<td>0.570</td>
</tr>
<tr>
<td><strong>Fiber (g/d)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>29.6 ± 1.4</td>
<td>28.9 ± 1.6</td>
<td>0.735</td>
</tr>
<tr>
<td>Intervention</td>
<td>37.5 ± 1.6</td>
<td>34.3 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>8.1 (4.7, 11.5)</td>
<td>5.2 (1.8, 8.6)</td>
<td>0.225</td>
</tr>
<tr>
<td><strong>Sodium (mmol/d)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2982.1 ± 206.5</td>
<td>3457.7 ± 291.5</td>
<td>0.190</td>
</tr>
<tr>
<td>Intervention</td>
<td>2023.9 ± 108.9</td>
<td>2669.2 ± 172.9</td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>−1144.1 (−1417.0, −871.2)</td>
<td>−602.7 (−875.6, −329.8)</td>
<td>0.007</td>
</tr>
</tbody>
</table>
Although dietary records are not a good measure of actual sodium intake, after adjustment for baseline sodium intake, there was a significant reduction in dietary sodium in the WELL group only. It is therefore likely that those following the WELL diet did have a lower intake of sodium. Subjects were advised to avoid added salt and obviously salty foods, and we found in a previous study (9) that this type of dietary advice can result in a decrease in 24-h urinary sodium of $\approx 30$ mmol in a weight-stable situation. In the present study, in which there was a reduction in energy intake, the reduction in sodium is likely to have been greater, but without having data on 24-h urine collections we cannot confirm this finding.

The decreases in BP in the present study were somewhat greater than in other studies, particularly the $5$–mm Hg decrease in DBP. A meta analysis of weight loss and BP indicated an average decrease in SBP and BDP of $4$ mm Hg in studies with energy restriction with or without exercise (15), whereas we found decreases of $8$ and $5$ mm Hg in SBP and DBP, respectively. Our results contrast with those of the recent large, multicenter Premier study (16). In that study, untreated subjects with mild hypertension who were randomly assigned to the DASH intervention achieved decreases of $11$ mm Hg in SBP and $7$ mm Hg in DBP with a $5$-kg weight loss over $6$ mo, and these decreases were not significantly different from those seen in the established care group, who had a similar weight loss.

Ours is the first study to assess the effect of a weight-loss intervention on home BP measurements rather than on investigator-measured BP. Some of the difference in BP response could be attributed to the different method of BP assessment. Home BP, measured at the same time of day, under the same conditions, shows reduced variability. Home BP measurement is now emerging as a preferred method of measuring BP (17), because it has been shown to share some of the advantages of ambulatory BP, that is, to have no “white coat” effect (18), to be more reproducible (19, 20), and to be more predictive of the presence and progression of organ damage than are office or clinic values (21). Because all our subjects used BP monitors for which their data were downloaded directly to a computer by the study staff, there was no possibility for errors in subject recording.
The results of the present study clearly show that targeted dietary advice (ie, to include ≥4 servings each of fruit and vegetables per day, 3 servings of nonfat dairy products per day, and 3 servings of fish and 1 serving of legumes per week and to avoid butter and added salt) combined with advice to walk 3 servings of fish and 1 serving of legumes per week and to avoid tables per day, 3 servings of nonfat dairy products per day, and calcium intakes.

REFERENCES


Plasma lycopene, other carotenoids, and retinol and the risk of cardiovascular disease in men¹–³

Howard D Sesso, Julie E Buring, Edward P Norkus, and J Michael Gaziano

ABSTRACT

Background: Emerging evidence suggests a possible role of lycopene in the primary prevention of cardiovascular disease (CVD).

Objective: We examined whether plasma lycopene concentrations in the Physicians’ Health Study were associated with CVD in a prospective, nested, case-control design.

Design: Baseline blood samples were collected starting in 1996. During a mean follow-up of 2.1 y, we identified 499 cases of CVD (confirmed myocardial infarction, stroke, CVD death, or revascularization procedures) and an equal number of men free of CVD and matched for age (± 69.7 y), follow-up time, and smoking status. We collected self-reported coronary disease risk factors and measured plasma carotenoids, retinol, lipids, and C-reactive protein.

Results: In matched analyses with additional adjustment for plasma total cholesterol and randomized treatment, the relative risks (RRs) of total CVD were 1.00 (reference), 0.92, 1.04, and 0.95 (for linear trend = 0.93). With multivariate adjustment, the RRs of total CVD were 1.00 (reference), 1.08, 0.94, and 1.03 (for linear trend = 0.98). For important vascular events (241 cases), excluding revascularization procedures, the multivariate RRs remained nonsignificant (P for linear trend = 0.50). Adding plasma carotenoids, lipids, or C-reactive protein to multivariate models had a minimal effect on the RRs of total CVD for plasma lycopene. Compared with lycopene, higher concentrations of plasma lutein/zeaxanthin and retinol suggested a moderate increase in CVD risk, whereas no association was found for β-cryptoxanthin, α-carotene, and β-carotene.

Conclusions: Higher plasma lycopene concentrations were not associated with the risk of CVD in this study of older men. Further evaluation in diverse populations is necessary.


KEY WORDS Lycopene, carotenoids, cardiovascular disease, prospective studies, nutrition, epidemiology

INTRODUCTION

In addition to a well-established inverse association between the intake of lycopene, a carotenoid without provitamin A activity, and prostate cancer (1), evidence also suggests a role of lycopene in the primary prevention of cardiovascular disease (CVD) (2–8). Because lycopene is predominantly found in just a few food sources, as tomato-based foods make up ≥80% of lycopene intake in the American diet (9), any benefits attributed to lycopene may lead to a straightforward intervention to increase intake. This provides an advantage for lycopene over other carotenoids, which are more ubiquitous in fruit and vegetables. Lycopene may enhance LDL degradation (10, 11), although mixed results from 3 dietary intervention studies have yet to verify the biological mechanism (11–13). In addition, lycopene may also be associated with the acute phase response in atherosclerosis (14) and is associated with C-reactive protein concentrations in 2 cross-sectional studies (15, 16).

We recently reported a significant association between plasma lycopene and a reduced risk of CVD in a nested case-control study of middle-aged and older women (17). In that study, there was a threshold effect above which plasma lycopene appeared to reduce the risk of CVD. Therefore, we sought to replicate these findings in a nested case-control study of older men from the Physicians’ Health Study (PHS). With no widely accepted biological mechanism to explain how lycopene may reduce the risk of CVD, we explored a priori the role of confounding of other carotenoids, lipids, and inflammatory markers with lycopene to explain any effect that may be observed for plasma lycopene (4, 18, 19).

SUBJECTS AND METHODS

Study population

The Physicians’ Health Study (PHS) began as a trial in 1982 of 22 071 men testing aspirin and β-carotene in the primary prevention of cancer and CVD (20, 21). On its scheduled completion

¹ From the Divisions of Preventive Medicine and Aging, Department of Medicine, Brigham & Women’s Hospital and Harvard Medical School, Boston, MA (HDS, JEB, and JMG); the Massachusetts Veterans Epidemiology Research and Information Center, VA Boston Healthcare System, Boston, MA (HDS, JEB, and JMG); the Department of Epidemiology, Harvard School of Public Health, Boston, MA (JEB); the Department of Ambulatory Care and Prevention, Harvard Medical School, Boston, MA (JEB); the Departments of Medical Research, Our Lady of Mercy Medical Center and Community and Preventive Medicine, New York Medical College, Bronx, NY (EPN).

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³ Reprints not available. Address correspondence to HD Sesso, Brigham and Women’s Hospital, 900 Commonwealth Avenue East, Boston MA 02215-1204. E-mail: hsesso@hsph.harvard.edu.

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in 1995, the PHS II was initiated as a randomized, double-blind, placebo-controlled, 2 × 2 × 2 factorial trial testing the benefits and risks of vitamin E, vitamin C, a multivitamin, and β-carotene in the primary prevention of cancer, CVD, and other chronic diseases in 14,641 male physicians aged ≥50 y (22), including 7641 original physicians from the PHS plus 7000 new physicians recruited into the trial. The β-carotene component of the trial was terminated in 2003, in part because of the continued lack of effect of β-carotene on cancer (21, 23) and CVD (21) and because there was no evidence of long-term side effects from >20 y of use. In total, there were 29,071 men randomly assigned into the PHS at either starting point in 1982 or 1995.

Fasting baseline blood samples were collected from PHS participants starting in 1996 and stored in liquid nitrogen until analyzed. For the present analyses, we conducted a prospective nested case-control study identifying 499 cases of CVD and an equal number of controls, all of whom were free of CVD at baseline and provided baseline blood samples starting in 1996 that could be assayed for lycopene. Cases included men who experienced a cardiovascular event after their baseline blood collection, defined as CVD death, nonfatal myocardial infarction (MI), nonfatal stroke, percutaneous transluminal coronary angioplasty, or coronary artery bypass graft. Study physicians conducted blinded reviews of all cases. CVD death was documented by convincing evidence of a cardiovascular mechanism from death certificates and medical records. The diagnosis of MI was confirmed by using World Health Organization criteria (24). A stroke was defined as a typical neurologic deficit, sudden or rapid in onset, lasting >24 h. Revascularization procedures were confirmed by hospital records. During a mean follow-up of 2.1 y, we identified 499 cases of CVD.

Each case of CVD was matched with a control according to age (±1 y), smoking status (never, former, or current smoker), follow-up time from baseline blood collection (±6 mo), and cohort designation (original or new physician in the PHS).

Blood assays

All investigators and laboratory personnel were blinded to the subject’s case-control status. Blood samples were handled identically and blindly through all stages of the blood collection, storage, retrieval, and analysis processes. Baseline plasma blood samples from cases and controls were thawed and assayed for total lycopene, other carotenoids, and retinol at Our Lady of Mercy Medical Center, Bronx, NY. All assays were quantified by reversed-phase HPLC after extraction and concentration by conventional methods (25). Internal standards (echinenone for carotenoids and retinyl propionate for retinol) were used to correct for recoveries of all samples that were analyzed, and the laboratory has participated in the US Quality Assurance Program. We also assayed plasma lipids, including total cholesterol and HDL cholesterol, using commercially available diagnostic kits (Sigma-Aldrich Chemical Co, St Louis, MO) and conventional methods (26, 27). Plasma total cholesterol was assayed because plasma lipoproteins are nonspecific carriers for all the carotenoids in plasma and, to date, total cholesterol appears to be the best way to control for confounding effects due to differences in lipoprotein concentrations between subjects (28). Finally, C-reactive protein was assayed by using a validated, high-sensitivity assay (Denka Seiken Company, Tokyo). On the basis of externally prepared control specimens, the laboratory accuracy is within 7% for each measured carotenoid, whereas the day-to-day and within-day precision (CV) for these assays was 5%.

Baseline covariates

The date of blood collection served as the start of follow-up for this study. Some, but not all, questionnaire information on other self-reported baseline risk factors for CVD was collected with the blood sample. We used the questionnaires that were completed closest to the time of blood collection, always within a few years, to ensure a complete assessment of confounding for our analyses. On these questionnaires, men provided self-reported data on age (in y), weight and height (converted to body mass index; in kg/m2), smoking status (categorized as never, former, or current), alcohol use (categorized as rarely or never, <1 drink/d, or ≥1 drink/d), frequency of exercise (categorized as rarely or never, <3 d/wk, 3–4 d/wk, or ≥5 d/wk), parental history of MI at <60 y of age (yes or no), history of hypertension (yes or no), history of diabetes (yes or no), and history of hypercholesterolemia (yes or no).

Data analyses

Men were first compared according to case-control status by using mean values or proportions of baseline coronary disease risk factors and biochemical markers. Measurements of plasma lycopene concentrations were divided into quartiles based on the overall distribution of plasma lycopene among the 499 controls. Coronary disease risk factors were also compared according to quartiles of plasma lycopene among the control population to assess potential confounding, with the use of ANOVA for continuous variables, Cochran-Armitage trend tests for dichotomous variables, or chi-square tests for categorical variables. Conditional logistic regression analyses generated the RRs and 95% CIs of future CVD for increasing quartiles of plasma lycopene concentrations, with the lowest quartile as the referent. Linear trends across quartiles of plasma lycopene concentrations were tested by using the median concentration for each quartile as an ordinal variable. Power calculations indicated that we had 54% and 98% power to detect a significant linear trend across quartiles, assuming corresponding RRs of 0.70 and 0.50 comparing the highest with the lowest quartile.

Models were first adjusted for randomized treatment assignments and plasma total cholesterol concentration; the next model added body mass index, exercise, alcohol consumption, parental history of MI at <60 y of age, hypertension, diabetes, and hypercholesterolemia. Additional models examined whether other biomarkers—including other carotenoids, HDL cholesterol, or C-reactive protein—added individually to a multivariate model confounded the association between plasma lycopene and CVD, possibly providing mechanistic evidence for any effect attributed to plasma lycopene.

To address a possible threshold effect above which plasma lycopene may be associated with the risk of CVD, as noted in a previous study of women (17), we also considered the association between men with plasma lycopene at or above the 90th percentile and the risk of CVD compared with those in the lowest quartile. Consistent with the previously reported findings for a stronger effect of plasma lycopene and important vascular events (limited to CVD death, MI, and stroke) (17), we repeated the analyses limited to 241 case-control pairs of important vascular events. We compared our results for plasma lycopene with those
for other carotenoids and retinol, with each biochemical marker divided into quartiles among the controls and entered into a separate model.

**RESULTS**

We first compared baseline characteristics for 499 cases of CVD and an equal number of matched controls who remained free of CVD (Table 1). As expected, cases of CVD tended to have a higher body mass index than did controls and were significantly more likely to have a history of hypertension, diabetes, hypercholesterolemia, and early parental history of MI. There were no significant differences in the frequencies of exercise and alcohol consumption between cases and controls (P > 0.05 for all). When matched for age and smoking status, no significant differences were observed between the concentrations of plasma carotenoids in cases and controls, including comparisons when individual concentrations of plasma lutein, zeaxanthin, and the combination of lutein and zeaxanthin were examined. CVD cases had significantly higher total cholesterol concentrations and lower HDL cholesterol concentrations than did controls (P < 0.05 for both). C-reactive protein concentrations were not significantly different (P = 0.66), whether compared as mean (P = 0.66) or log-transformed (P = 0.38) concentrations (data not shown).

Among the 499 controls, plasma lycopene was significantly (P < 0.05 for all) correlated with plasma concentrations of β-cryptoxanthin (Spearman r = 0.16), lutein/zeaxanthin (r = 0.27), α-carotene (r = 0.24), and β-carotene (r = 0.22). There was no significant difference in the Spearman correlation coefficients when plasma lycopene was compared with either lutein alone or zeaxanthin alone (r = 0.27 for both). Plasma lycopene was less strongly correlated with plasma retinol, with a Spearman correlation of 0.11 (P = 0.018). Total cholesterol, a nonspecific carrier of lycopene and other carotenoids, was most strongly and significantly correlated with plasma lycopene (Spearman r = 0.26) compared with values of 0.17 for lutein/zeaxanthin, 0.11 for β-cryptoxanthin, 0.09 for retinol, and 0.06 for both α- and β-carotene. When we calculated total cholesterol–adjusted Spearman correlations between plasma lycopene and other carotenoids, the correlations were only modestly reduced in magnitude and remained significant. Finally, plasma lycopene was
negatively correlated with plasma C-reactive protein concentrations (Spearman $r = -0.11$, $P = 0.016$).

The plasma lycopene quartile cutoffs were defined as $\leq 6.4$, 6.4–9.3, 9.3–12.7, and $> 12.7 \mu g/dL$ based on the distribution of values in the 499 men who were free of CVD. A comparison of baseline characteristics across these quartiles of plasma lycopene in the 499 controls is shown in Table 2. Aside from significant inverse associations between plasma lycopene and both age and history of hypertension, other lifestyle and clinical risk factors did not differ appreciably across quartiles of plasma lycopene. Plasma carotenoids increased significantly ($P < 0.05$ for all) with increasing plasma lycopene, with the exception of plasma $\beta$-carotene. Total cholesterol concentrations increased, and HDL-cholesterol concentrations decreased with higher concentrations of plasma lycopene ($P < 0.001$ for both). C-reactive protein decreased with increasing quartiles of plasma lycopene but was not statistically significant as either an untransformed ($P = 0.31$) or log-transformed ($P = 0.17$) variable.

A series of models calculating the relative risks (RRs) and 95% CIs of either total CVD or important vascular events, based on quartiles of plasma lycopene, are provided in Table 3. Neither the crude nor the multivariate models that were further adjusted for lifestyle and clinical factors showed a significant association between plasma lycopene and the risk of total CVD ($P$ for linear trend $> 0.05$ for both). There was minimal attenuation of the RRs with control for potential confounders. Men with baseline plasma lycopene concentrations at or above the 90th percentile ($\geq 15.9 \mu g/dL$) also had no association with the risk of CVD, as the multivariate RR was 1.25 (95% CI: 0.70, 2.23). When we considered analyses restricted to the 241 case-control pairs of men with important vascular events (MI, stroke, and CVD death), the multivariate RRs for increasing quartiles of plasma lycopene were 1.00 (reference), 1.02 (0.51–2.04), 0.93 (0.47–1.85), and 0.80 (0.39–1.64) ($P$ for linear trend = 0.50). As before, this lack of an association extended to men at or above the 90th percentile of plasma lycopene. For secondary analyses of increasing quartiles of plasma lycopene with the risk of MI (117 case-control pairs), the multivariate RRs were 1.00 (reference), 0.91, 0.84, and 0.68 ($P$ for linear trend = 0.49) with wide 95% CIs. For stroke (71 case-control pairs), the RRs were 1.00 (reference), 2.66, 0.67, and 1.36 ($P$ for linear trend = 0.91), again with extremely wide 95% CIs that limited our ability to dismiss chance. We also explored whether the additional adjustment for plasma carotenoids, retinol, HDL cholesterol, or C-reactive protein attenuated the association between plasma lycopene and the risk of CVD.
TABLE 3
Relative risks (RRs) and CIs of total cardiovascular disease (499 case-control pairs) and important vascular events (241 case-control pairs; limited to myocardial infarction, stroke, and cardiovascular disease death) according to quartile of plasma lycopene

| Quartile of plasma lycopene | 1 (≤6.4 µg/dL) | 2 (6.4–9.3 µg/dL) | 3 (9.3–12.7 µg/dL) | 4 (>12.7 µg/dL) | P for linear trend | ≥90th Percentile (≥15.9 µg/dL)
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Median plasma lycopene (µg/dL)</td>
<td>4.6</td>
<td>7.9</td>
<td>11.0</td>
<td>15.3</td>
<td>18.6</td>
<td></td>
</tr>
<tr>
<td>Total cardiovascular disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of cases</td>
<td>121</td>
<td>126</td>
<td>126</td>
<td>124</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>No. of controls</td>
<td>123</td>
<td>126</td>
<td>126</td>
<td>124</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Crude RR</td>
<td>1.00 (ref)</td>
<td>0.92 (0.64, 1.32)</td>
<td>1.04 (0.72, 1.49)</td>
<td>0.95 (0.65, 1.40)</td>
<td>0.93</td>
<td>1.13 (0.69, 1.84)</td>
</tr>
<tr>
<td>Multivariate-adjusted RR</td>
<td>1.00 (ref)</td>
<td>1.08 (0.71, 1.66)</td>
<td>0.94 (0.61, 1.44)</td>
<td>1.03 (0.65, 1.64)</td>
<td>0.98</td>
<td>1.25 (0.70, 2.23)</td>
</tr>
<tr>
<td>+ Plasma β-cryptoxanthin</td>
<td>1.00 (ref)</td>
<td>1.07 (0.70, 1.65)</td>
<td>0.93 (0.60, 1.44)</td>
<td>1.02 (0.64, 1.62)</td>
<td>0.93</td>
<td>1.23 (0.68, 2.21)</td>
</tr>
<tr>
<td>+ Plasma α-carotene</td>
<td>1.00 (ref)</td>
<td>1.10 (0.71, 1.69)</td>
<td>0.95 (0.61, 1.46)</td>
<td>1.05 (0.66, 1.66)</td>
<td>0.99</td>
<td>1.27 (0.70, 2.27)</td>
</tr>
<tr>
<td>+ Plasma β-carotene</td>
<td>1.00 (ref)</td>
<td>1.09 (0.71, 1.69)</td>
<td>0.94 (0.61, 1.46)</td>
<td>1.04 (0.65, 1.66)</td>
<td>0.99</td>
<td>1.26 (0.70, 2.25)</td>
</tr>
<tr>
<td>+ Plasma retinol</td>
<td>1.00 (ref)</td>
<td>1.06 (0.69, 1.62)</td>
<td>0.93 (0.60, 1.43)</td>
<td>1.00 (0.63, 1.60)</td>
<td>0.90</td>
<td>1.22 (0.68, 2.19)</td>
</tr>
<tr>
<td>+ Plasma HDL cholesterol</td>
<td>1.00 (ref)</td>
<td>1.17 (0.76, 1.80)</td>
<td>0.94 (0.61, 1.47)</td>
<td>1.00 (0.62, 1.60)</td>
<td>0.80</td>
<td>1.21 (0.67, 2.18)</td>
</tr>
<tr>
<td>+ Plasma C-reactive protein</td>
<td>1.00 (ref)</td>
<td>1.07 (0.69, 1.65)</td>
<td>1.03 (0.66, 1.60)</td>
<td>1.02 (0.63, 1.63)</td>
<td>0.99</td>
<td>1.26 (0.69, 2.29)</td>
</tr>
<tr>
<td>Important vascular events</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude RR</td>
<td>1.00 (ref)</td>
<td>0.93 (0.55, 1.57)</td>
<td>1.28 (0.74, 2.21)</td>
<td>0.94 (0.53, 1.67)</td>
<td>0.88</td>
<td>1.02 (0.53, 1.99)</td>
</tr>
<tr>
<td>Multivariate-adjusted RR</td>
<td>1.00 (ref)</td>
<td>1.02 (0.51, 2.04)</td>
<td>0.93 (0.47, 1.85)</td>
<td>0.80 (0.39, 1.64)</td>
<td>0.50</td>
<td>1.11 (0.49, 2.51)</td>
</tr>
</tbody>
</table>

1 Linear trends across quartiles of plasma lycopene concentrations were tested by using the median concentration for each quartile as an ordinal variable.
2 Men in the ≥90th percentile of plasma lycopene were included in the upper (4th) quartile.
3 Matched for age and smoking status and adjusted for plasma total cholesterol and randomized treatment assignments.
4 Reference.
5 Adjusted for the covariates listed above plus BMI, exercise, alcohol, parental history of myocardial infarction <60 y of age, diabetes, hypertension, and high cholesterol.
6 Other carotenoids and retinol were added separately into the model.

In general, there was little indication that any of the aforementioned plasma markers had an effect on any of the reported RRs of CVD (P for linear trend remained > 0.05 for all). Separate, additional control for either plasma lutein or plasma zeaxanthin did not affect the RRs of CVD for plasma lycopene either. Replication of these models for important vascular events resulted in a parallel lack of confounding on the RRs.

For comparison, we then examined the multivariate RRs for plasma lycopene versus other carotenoids and retinol for the risk of CVD based on quartiles of plasma carotenoids in the 499 controls in Table 4. Confounding was minimal when crude and multivariate models for each plasma marker were compared (data not shown). Of note, men in higher quartiles of plasma lutein/zeaxanthin had nonsignificant but persistent elevations in

TABLE 4
Multivariate relative risks (RRs) and 95% CIs of cardiovascular disease (499 case-control pairs) for a comparison of quartile of plasma lycopene with other plasma carotenoids and retinol in separate models

<table>
<thead>
<tr>
<th>Quartile of plasma biomarker</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>P for linear trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma lycopene (µg/dL)²</td>
<td>4.6</td>
<td>7.9</td>
<td>11.0</td>
<td>15.3</td>
<td>0.98</td>
</tr>
<tr>
<td>Multivariate-adjusted RR</td>
<td>1.00 (ref)⁴</td>
<td>1.08 (0.71, 1.66)</td>
<td>0.94 (0.61, 1.44)</td>
<td>1.03 (0.65, 1.64)</td>
<td>0.98</td>
</tr>
<tr>
<td>Plasma β-cryptoxanthin (µg/dL)²</td>
<td>3.5</td>
<td>6.8</td>
<td>11.2</td>
<td>20.9</td>
<td></td>
</tr>
<tr>
<td>Multivariate-adjusted RR</td>
<td>1.00 (ref)</td>
<td>0.84 (0.55, 1.29)</td>
<td>0.87 (0.58, 1.31)</td>
<td>0.85 (0.55, 1.32)</td>
<td>0.61</td>
</tr>
<tr>
<td>Plasma lutein/zeaxanthin (µg/dL)²</td>
<td>11.7</td>
<td>16.9</td>
<td>22.2</td>
<td>31.7</td>
<td></td>
</tr>
<tr>
<td>Multivariate-adjusted RR</td>
<td>1.00 (ref)</td>
<td>1.31 (0.84, 2.04)</td>
<td>1.21 (0.76, 1.95)</td>
<td>1.41 (0.86, 2.31)</td>
<td>0.28</td>
</tr>
<tr>
<td>Plasma retinol (µg/dL)⁷</td>
<td>48.1</td>
<td>61.3</td>
<td>70.8</td>
<td>88.3</td>
<td></td>
</tr>
<tr>
<td>Multivariate-adjusted RR</td>
<td>1.00 (ref)</td>
<td>1.47 (0.92, 2.33)</td>
<td>1.22 (0.77, 1.93)</td>
<td>1.74 (1.06, 2.84)</td>
<td>0.06</td>
</tr>
<tr>
<td>Plasma α-carotene (µg/dL)²</td>
<td>2.3</td>
<td>4.6</td>
<td>7.2</td>
<td>12.2</td>
<td></td>
</tr>
<tr>
<td>Multivariate-adjusted RR</td>
<td>1.00 (ref)</td>
<td>1.06 (0.71, 1.58)</td>
<td>0.79 (0.51, 1.32)</td>
<td>1.18 (0.76, 1.82)</td>
<td>0.52</td>
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<tr>
<td>Plasma β-carotene (µg/dL)²</td>
<td>9.9</td>
<td>18.0</td>
<td>27.2</td>
<td>49.1</td>
<td></td>
</tr>
<tr>
<td>Multivariate-adjusted RR</td>
<td>1.00 (ref)</td>
<td>0.69 (0.45, 1.06)</td>
<td>0.74 (0.48, 1.15)</td>
<td>0.85 (0.56, 1.29)</td>
<td>0.77</td>
</tr>
</tbody>
</table>

¹ Linear trends across quartiles of plasma lycopene concentrations were tested by using the median concentration for each quartile as an ordinal variable.
² Median value for each plasma biomarker for that quartile.
³ Matched for age and smoking status and adjusted for plasma total cholesterol, randomized treatment assignments, BMI, exercise, alcohol, parental history of myocardial infarction <60 y of age, diabetes, hypertension, and high cholesterol.
⁴ Reference.
the risk of CVD compared with those in the lowest quartile. Separately, the multivariate RRs of CVD for the second through fourth quartiles of plasma lutein were 1.19 (0.76, 1.87), 1.21 (0.75, 1.94), and 1.30 (0.79, 2.14) \(P\) for linear trend = 0.37; for plasma zeaxanthin, the RRs were 1.28 (0.82, 2.00), 1.23 (0.77, 1.97), and 1.40 (0.85, 2.29) \(P\) for linear trend = 0.28. Finally, elevations in plasma retinol were also associated with a borderline significant increased risk of CVD \(P\) for linear trend = 0.06.

**DISCUSSION**

We found no overall association between increasing concentrations of plasma lycopene and the risk of CVD in this nested case-control study of older male physicians. This observed lack of association persisted when we also considered concentrations of plasma lycopene of at least the 90th percentile, and when the analyses were limited to only the most important vascular events (MI, stroke, and CVD death). There was no evidence of substantial confounding by major lifestyle and clinical risk factors of CVD. Because we found no overall association between plasma lycopene and CVD, however, our examination of confounding by other carotenoids, lipids, and inflammatory markers could not be reliably addressed.

These results were inconsistent with other findings for plasma lycopene and other carotenoids and the risk of CVD, using different PHS data. Two separate nested case-control studies of MI and ischemic stroke used the earlier 1982 baseline blood samples from the PHS. For plasma lycopene and MI, RRAs for increasing quintiles of plasma lycopene were 1.00 (reference), 1.35, 1.41, 1.54, and 1.43 \(P\) for linear trend = 0.13 (29). In contrast, other data based on the earlier 1982 baseline blood samples for a nested case-control study of carotenoids and ischemic stroke showed a potential inverse association for lycopene (30). Specifically, the RRAs (95% CIs) of stroke for increasing quintiles of lycopene were 1.00 (reference), 0.66 (0.36, 1.23), 0.54 (0.29, 1.00), 0.55 (0.29, 1.05), and 0.72 (0.38, 1.37). A comparison of the upper 4 with the lowest quintiles showed a 39% risk reduction compared with a nonsignificant increased risk of total stroke in this analysis. Three explanations may account for these differences. First, the PHS subjects were \(\sim\)10 years older in this study. Increasing age was inversely and strongly associated with plasma lycopene concentrations in this and other studies (31, 32). Furthermore, the relevant determinants of plasma lycopene may vary by age (31, 33, 34). Median plasma lycopene concentrations among the PHS controls were considerably lower in these analyses (9.3 \(\mu\)g/dL) in older men compared with the earlier PHS analyses (36.6 or 39.9 \(\mu\)g/dL). Second, the noted differences among studies may have been because 2 different laboratories using different methods conducted the carotenoid assays. Third, although matching schemes were similar across all 3 nested case-control studies, the random nature of selecting cases and controls may also have accounted for the inconsistent findings for plasma lycopene.

In addition, the data from the present study in men counter the strong inverse association found when we examined plasma lycopene for the risk of CVD in a nested case-control study among middle-aged and older female health professionals from the Women’s Health Study (WHS) (17). Although chance cannot be ruled out as an explanation for the present findings, there were other differences between the PHS and WHS study populations that may explain these discrepant findings. First, the PHS had more limited follow-up time (\(\bar{x} = 2.1\) years) compared with the WHS (\(\bar{x} = 4.9\) years). As a result, the present study may have identified more men with preexisting atherosclerosis that would imminently become manifest as CVD. However, our thorough control for potential confounders should help to minimize such confounding. A second possible explanation for the differences in results from this study compared with the WHS is that the median concentration of plasma lycopene among men in the PHS (9.3 \(\mu\)g/dL) was lower than that among women in the WHS (16.5 \(\mu\)g/dL). This difference may perhaps reflect not only established sex differences in plasma lycopene and carotenoid concentrations but also the fact that these men were on average older (PHS: 69.7 years; WHS: 58.8 years). The broad range of plasma lycopene concentrations observed in the PHS is at the low end of the range of other reported plasma lycopene concentrations (4, 5, 15, 32, 34–40).

As for other studies that have examined plasma lycopene and the risk of CVD, higher adipose tissue concentrations of lycopene are associated with reduced intimal wall thickness (2, 3), although in a Finnish cohort this was found in men but not in women (39). Several other studies report possible inverse associations of serum lycopene concentrations and the risk of MI (4, 40), CVD (6, 7, 40), carotid atherosclerosis (5, 8), and aortic atherosclerosis (41).

Lycopene has garnered promise for a role in the primary prevention of CVD because it is one of the most potent singlet oxygen quenchers, which suggests that it may have comparatively stronger antioxidant properties than its related carotenoids (42). An apparent role in reducing LDL oxidation (10–12) has not necessarily been substantiated with reductions in LDL cholesterol (11), although more studies are needed in this area. The observation that C-reactive protein concentrations were lower among male controls in higher lycopene quartiles is consistent with the findings from other studies (15, 16) for a potential role in mitigating atherogenesis (14).

Among study limitations, we relied on a single baseline measurement of plasma lycopene, raising the possibility of regression to the mean, biasing our RRAs toward the null hypothesis and thus underestimating the observed risk reductions. The long-term stability of plasma lycopene has not been tested in our blood samples; however, other studies support the stability of lycopene and other biochemical markers used in the present study (43, 44). We also relied on a composite endpoint for total CVD including CVD death, MI, stroke, and revascularization. Heterogeneity in the results by specific CVD endpoints cannot be ruled out because we had limited power to assess any single outcome. Finally, residual confounding by diet and other risk factors may still be present. However, besides matching for age, smoking, and follow-up time, we believe that we have comprehensively controlled for other coronary risk factors. In addition, in our previous study among women, dietary factors did not strongly attenuate the observed RRAs (17).

In conclusion, higher plasma lycopene concentrations were not associated with a reduced risk of CVD in middle-aged and older men. It is critical for additional research not only to carefully examine the association between both plasma and dietary lycopene with the risk of CVD but also to conduct clinical studies that directly test the proposed mechanisms through which lycopene may play a role in the prevention of CVD.
We acknowledge the crucial contributions of the entire staff of the PHS and are indebted to the 29,071 dedicated and committed participants who were randomly assigned into the PHS starting in either 1982 or 1995.

HDS conceived and designed the study, analyzed and interpreted the data, drafted the article, and obtained funding. JEB critically revised the article, collected and assembled the data, and obtained funding. EPN critically revised the article and collected and assembled the data. JMG conceived and designed the study, analyzed and interpreted the data, critically revised the article, and obtained funding. The authors had no personal or financial interest in any company or organization sponsoring the research.

REFERENCES

Dietary antioxidants and fat are associated with plasma antibody titers to heat shock proteins 60, 65, and 70 in subjects with dyslipidemia

Majid Ghayour-Mobarhan, Susan A New, David J Lamb, Bryan J Starkey, Callum Livingstone, Tim Wang, Nandita Vaidya, and Gordon A Ferns

ABSTRACT

Background: The heat shock proteins (HSPs) are protein chaperones. Higher titers of antibody to HSPs (anti-HSPs) have been reported in atherosclerosis, which may contribute to immunooactivation in this process.

Objective: We investigated whether dietary antioxidants and fat intake are associated with changes in anti-HSP titers in dyslipidemic subjects.

Design: Patients (n = 238) were recruited from hospital lipid clinics. Control subjects (n = 188) were recruited from university and hospital employees. Food-frequency questionnaires were used to estimate dietary antioxidants and fat.

Results: Dyslipidemic patients had significantly higher titers of anti-HSPs than did control subjects; expressed in medians and interquartile ranges of absorbance units, anti-HSP-60 titers were 0.27 (0.18–0.37) and 0.22 (0.16–0.30), anti-HSP-65 titers were 0.45 (0.28–0.79) and 0.31 (0.22–0.50), and anti-HSP-70 titers were 0.22 (0.17–0.30) and 0.19 (0.13–0.27), respectively. Median and interquartile ranges of serum concentrations of C-reactive protein were 1.25 (0.17–0.30) and 0.19 (0.13–0.27), anti-HSP-70 titers were 0.22 (0.18–0.37) and 0.22 (0.16–0.30), anti-HSP-65 titers were 0.45 (0.28–0.79) and 0.31 (0.22–0.50), and anti-HSP-60 titers were 0.27 (0.18–0.38) were also significantly higher in patients than in control subjects, respectively. In dyslipidemic patients, the major dietary predictors of the variability in anti-HSP-60 titers were vitamin C (P = 0.005), vitamin E (P = 0.04), and total fat (P = 0.009) intakes; for anti-HSP-65 titers, vitamin C was the major predictor (P = 0.002). These findings remained significant after adjustment for confounding factors.

Conclusions: Anti-HSP-60, -65, and -70 titers are significantly higher in dyslipidemic patients with or without established coronary disease. Our data indicate an association between dietary constituents and the immune response to HSPs in dyslipidemic subjects.

KEY WORDS Heat shock proteins, HSP-60, -65, and -70, antibody titers, dietary intake, antioxidants, fat

INTRODUCTION

The heat shock proteins (HSPs) are a family of 20–25 molecules expressed by cells in response to stresses such as high temperature, free radicals, shear stress, and toxins, including oxidized LDL cholesterol. They are involved in the renaturation of damaged proteins, allowing them to refold into their native conformation. It has been proposed that, because the structure of HSPs is highly conserved across species, the immune response mounted against bacterial HSPs may result in an immune response that has the potential to target endogenous HSPs and cause complement-mediated endothelial injury and thus to accelerate atherogenesis. Antibody titers to HSP-60, -65, and -70 have been reported to be associated with coronary risk factors, increased risk of cardiovascular disease, the severity of cardiovascular and vascular endpoints in patients with established disease, and high concentrations of HSP-65 enhances the atherogenic process.

There is substantial evidence that protein-calorie malnutrition and some specific nutrient deficiencies have adverse effects on the immune system. Alterations in the quality and quantity of dietary fat and abnormalities in lipid metabolism have been reported to influence immune responses. In vivo and in vitro studies showed that fatty acids can modulate the immune system, and animal and human studies showed the importance of vitamin E and zinc in maintaining immune function.

The effects of dietary constituents on HSPs have been studied in animal models. Kelly et al showed that vitamin E deprivation for 16 wk combined with exercise for 8 wk induced HSP-72 expression in female rats. Andrus et al showed that cyclosporine A–induced expression of HSP-70 by rat hepatocytes, which is mediated by the...
release of reactive oxygen species, is significantly reduced in the presence of vitamin E in vitro. It is interesting, however, that there were previous reports that vitamin C induced an increase in baseline expression of HSP-60 and -70 in vitro, but its mechanisms of action are unclear (23).

Romano et al (15) reported that a diet rich in saturated fatty acids induces the expression of HSP-25, -60, and -70 in mice splenic lymphocytes. We also showed that serum anti-HSP-65 titers rise in rabbits fed a high-cholesterol diet (24). To our knowledge, no studies have yet examined the association between dietary intake and HSP antibody titers in humans. Thus, the principal aim of our study was to investigate whether dietary factors are associated with HSP antibody titers in patients with dyslipidemia.

SUBJECTS AND METHODS

Study design and subject selection

Two hundred thirty-eight patients were recruited from the lipid clinics at the local hospital (the Royal Surrey County Hospital, Guildford, United Kingdom). One hundred eighty-eight control subjects were employees at the University of Surrey or the Royal Surrey County Hospital. Eighty-two patients were obese [body mass index (in kg/m²) >30]; 42 were diabetic (fasting plasma glucose concentration >7 mmol/L); 55 had established coronary artery disease (CAD), and 186 were hypertensive. Of the latter group, 76 had systolic blood pressure (SBP) ≥160 mm Hg or diastolic blood pressure (DBP) ≥100 mm Hg. One hundred ten patients had SBP between 130 and 160 mm Hg or DBP between 85 and 100 mm Hg. One hundred seventy-six patients had hypertension (SBP ≥160 mm Hg or DBP ≥100 mm Hg). Forty-two and 54 of the patients had a calculated 10-y coronary risk of >30% and 20–30%, respectively (risk calculated with the use of the PROCAM algorithm; 25), and 142 patients had metabolic syndrome by National Cholesterol Education Program Adult Treatment Panel III criteria (26). Patients with a history of established CAD included 9 with unstable angina, 15 with previous myocardial infarction, 10 with a history of coronary artery bypass grafting, and 13 with a history of angioplasty. Fifteen patients had undergone a coronary artery bypass graft after a myocardial infarction, and 5 had undergone angioplasty after a myocardial infarction. Fifty-four subjects were excluded from the control group because they were obese (n = 33), had metabolic syndrome (n = 9), or were on medication (n = 11; Table 1).

| TABLE 1 |
| Characteristics and medication use of dyslipidemic patients and control subjects† |
| n (%) |

**Patients**

| Obese, BMI >30 [n (%)] | 82 (35) |
| Type 2 diabetes, fasting blood glucose >7 mmol/L [n (%)] | 42 (18) |
| Duration of treatment for diabetes (mo) | 13 (0–51)² |
| Established coronary heart disease [n (%)] |
| Unstable angina | 9 (4) |
| MI | 15 (6) |
| CABG | 10 (4) |
| Angioplasty | 13 (6) |
| Angioplasty or CABG after MI | 8 (3) |
| Hypertension [n (%)] | 186 (79) |
| High blood pressure (SBP ≥160 mm Hg or DBP ≥100 mm Hg) | 76 (32) |
| Duration of treatment for hypertension (mo) | 7.5 (0–72) |
| Moderate blood pressure (SBP 130–160 mm Hg or DBP 85–100 mm Hg) | 110 (46) |
| Duration of treatment for hypertension (mo) | 0.0 (0–46) |
| Hypertriglyceridemia, serum triglycerides >1.8 mmol/L [n (%)] | 176 (74) |
| Hypercholesterolemia, serum total cholesterol >5.2 mmol/L [n (%)] | 216 (92) |
| Duration of statin therapy (mo) | 9.0 (0–43) |
| Duration of fibrate therapy (mo) | 0.0 (0–16) |
| Calculated 10-y coronary risk >30%³ | 54 (23) |
| Calculated 10-y coronary risk between 20% and 30%³ | 142 (60) |
| Metabolic syndrome⁴ | 135 (71) |
| Healthy [n (%)] | 135 (71) |
| Excluded [n (%)] | 53 (29) |
| Obese | 53 (29) |
| Metabolic syndrome | 33 (18) |
| Taking medications | 9 (4) |

¹ SBP, systolic blood pressure; DBP, diastolic blood pressure; MI, myocardial infarction; CABG, coronary artery bypass graft.
² Median; interquartile range for duration of treatment in parentheses (all such values).
³ Calculated with the PROCAM algorithm (25).
⁴ Defined according to National Cholesterol Education Program Adult Treatment Panel III criteria (26).
Each patient gave written informed consent to participate in the study. The study protocol was approved by the South-West Surrey Research Ethics Committee and the Advisory Committee of Surrey University.

Current dietary intakes and estimation of antioxidants and fat

Dietary intakes over the previous 12 mo were assessed by using a food-frequency questionnaire (FFQ) as previously detailed (27, 28). In brief, the FFQ was developed and validated against 7-d weighed records (29) and biochemical markers of antioxidant status (30), and its short- and long-term reproducibility was tested (31). During the initial interview, subjects were instructed in filling out the FFQ, and completed FFQs were checked for inaccuracies and inconsistencies at subsequent interviews.

Anthropometric and other measurements

All subjects were measured for height (in cm) and were weighed (in kg) with the use of a stand-on bioelectrical impedance analyzer (Tanita-305 body fat analyzer; Tanita Corp, Tokyo). The latter was also used to estimate percentage body fat. Body mass index was calculated as described above.

Waist and hip measurements were taken to the nearest millimeter as described previously (32). Blood pressure measurements were made with the use of an automated device (DINAMAP compact monitor, model TS; Critikon, Tampa, FL).

Blood sampling

Blood samples were collected between 0830 and 1030 after a 12-h fast by venipuncture of the antecubital vein. For the anti-HSP assays, blood samples were drawn into EDTA-containing tubes (Vacutainer; Becton Dickinson, Cowley, United Kingdom), which were centrifuged at 1000 × g for 15 min at 4 °C, and plasma aliquots were stored at −80 °C until the day on which HSP antibody titers were measured. Samples for lipid profile and measurement of serum vitamin E and high-sensitivity C-reactive protein (hs-CRP) were taken into plain Vacutainer tubes, and those for measurement of glucose were taken into Vacutainer tubes containing fluoride-oxalate. All chemicals were obtained from Sigma Chemical Co (Poole, United Kingdom) unless stated otherwise.

Analytic methods

Lipid profiles, high-sensitivity C-reactive protein, and blood glucose

A fasting lipid profile, comprising total cholesterol, triglycerides, and HDL cholesterol, was obtained for each patient. With the use of the formula of Friedewald et al (33), LDL cholesterol was calculated for all subjects except the patients with serum triglyceride concentrations >4.0 mmol/L. Lipids, hs-CRP, and glucose were measured by routine methods with a Bayer Advia 1650 analyzer (Bayer, Newbury, United Kingdom).

Serum vitamin E

Serum vitamin E was measured by HPLC. Briefly, 200 µL of an internal standard (10 µg δ-tocopherol/mL isopropyl alcohol) was added to 200 µL serum and mixed by vortex (34). Aqueous ammonium sulfate (3.9 mol/L) was added (200 µL), and the solution was again mixed by vortex. After centrifugation (1000 × g for 5 min), 50 µL of supernatant was used for analysis using a 150 × 4.6-mm Prodigy 50-µm ODS2 column (Phenomenex Ltd, Macclesfield, United Kingdom) with methanol as mobile phase and detection at 294 nm. At a flow rate of 1.4 mL/min, the retention time for internal standard and vitamin E was 5.2 and 6.6 min, respectively. Vitamin E standard and quality-control material were obtained from BioRad Laboratories Ltd (Hemel Hempstead, United Kingdom).

Heat shock protein antibody titers

Plasma HSP antibody titers were measured by using in-house enzyme-linked immunosorbent assays. In brief, a 96-well microtiter plate (Nunc Immunoplate Maxisorp; Scientific Laboratory Supplies Ltd, Nottingham, United Kingdom) was coated with human recombinant HSP-60, -65, or -70 by adding 10 ng recombinant HSP in phosphate-buffered saline (PBS) to the wells of a microtiter plate and incubating overnight at 4 °C. Plates were washed with PBS, blocked with Superblock (Pierce & Wariner, Chester, United Kingdom), and washed 3 times with PBS containing 0.05% (by vol) Tween-20. Plasma samples were diluted 1:15 with PBS containing 0.1% Tween-20 and 1% bovine serum albumin (PBT; Sigma-Aldrich Inc, Poole, United Kingdom), and 100 µL/well in quadruplicate was incubated for 30 min at 37 °C. After washing, bound HSP antibodies were detected by the addition of peroxidase-conjugated goat anti-human immunoglobulin G, which was diluted 1:100 with PBT. After washing with PBS/Tween-20, o-phenylenediamine (0.04%) [dissolved in 0.05 mol citrate/L with 0.1 mol phosphate buffer/L (pH 5) and containing 10 µL of a 30% solution of H2O2/25 mL] was added and incubated for 5 min at room temperature. The reaction was terminated by the addition of 3 mol hydrochloric acid/L. The absorbance was read at 492 nm by using a plate reader with GENESIS 2 software (version 2; Life Sciences, Basingstoke, United Kingdom) (35).

Statistical analysis

Statistical analysis was undertaken with the use of MINITAB software (release 13; Minitab Inc, State College, PA), with determination of descriptive statistics (ie, means, medians, SEMs, and interquartile ranges) for all variables. Data were assessed for normality by using the Kolmogorov-Smirnov test. Between-group comparisons of biochemical variables were assessed by analysis of variance. Categorical data were compared by using Fisher’s exact test or chi-square test. Values were expressed as means ± SEMs or medians and interquartile ranges (for nonnormally distributed data). Analysis of covariance was used to assess differences after adjustment for important confounding factors, such as age and physical activity. The hs-CRP concentrations and plasma titers of HSP antibody were found to be nonnormally distributed and were therefore logarithmically transformed before parametric analysis.

Stepwise multiple regression analysis was used to predict whether the anti-HSP titers were related to dietary antioxidants or fat. To enable adjustment for potential confounding factors, we entered into the equation the factors age, sex, obesity, metabolic syndrome or accumulating features of metabolic syndrome, diabetes mellitus, smoking, hypertriglyceridemia, blood pressure,
established CAD, calculated 10-y coronary risk factors, and drug treatment. A P value of < 0.05 was considered significant.

RESULTS

Descriptive data

There was a high frequency of obesity (35%), type 2 diabetes (18%), hypertension (79%), and positive smoking habit (18%) in the patient group, findings that are typical of a lipid clinic population. As expected among persons attending such a clinic, serum fasting triacylglycerol, plasma glucose, and total- and LDL-cholesterol concentrations were significantly higher for patients than for control subjects. Anthropometric indexes including waist size, waist-hip ratio, body mass index, and percentage body fat were also significantly higher in the patients than in the control subjects (Table 2). Although current smoking habits did not differ significantly between patients and control subjects, a significantly greater proportion of the patients were former smokers (Table 2). The patients were also significantly older than the control subjects.

Heat shock protein antibody titers

Dyslipidemic patients had significantly higher anti-HSP-60, -65, and -70 titers than did the control subjects (Table 3). The results remained the same after adjustment for age. A strong correlation between titers of anti-HSP-60, -65, and -70 (P = 0.001) was observed in patient and control groups.

Serum high-sensitivity C-reactive protein concentrations

Serum hs-CRP concentrations were significantly higher in the dyslipidemic patients than in the control subjects (Table 3). The results remained the same after adjustment for age. No significant association between HSP antibody titers and serum hs-CRP concentrations was observed in the patient or control groups (P > 0.05).

TABLE 3

Comparison of anti-heat shock protein (anti-HSP) titers and serum vitamin E and high-sensitivity C-reactive protein (hs-CRP) concentrations between dyslipidemic patients and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 238)</th>
<th>Control subjects (n = 135)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HSP titers (absorbance units)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP-60</td>
<td>0.27 (0.18–0.37)²</td>
<td>0.22 (0.16–0.30)</td>
<td>0.002</td>
</tr>
<tr>
<td>HSP-65</td>
<td>0.45 (0.28–0.79)³</td>
<td>0.31 (0.22–0.50)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HSP-70</td>
<td>0.22 (0.17–0.30)⁴</td>
<td>0.19 (0.13–0.27)</td>
<td>0.021</td>
</tr>
<tr>
<td>Serum hs-CRP concentrations (mg/L)</td>
<td>1.25 (0.42–3.26)⁵</td>
<td>0.58 (0.17–1.42)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum vitamin E concentrations (mg/L)</td>
<td>16.36 ± 0.31⁵⁵</td>
<td>14.08 ± 0.38</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ratio of vitamin E to total cholesterol</td>
<td>2.75 ± 0.05</td>
<td>2.60 ± 0.06</td>
<td>0.08</td>
</tr>
</tbody>
</table>

1 Mean; median and interquartile range in parentheses (all such values).
2 Median; interquartile range in parentheses (all such values).
3 P < 0.01.
4 P < 0.001.
5 P < 0.05.

TABLE 2

Clinical and biochemical measurements in dyslipidemic patients and control subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Patients (n = 288)</th>
<th>Control subjects (n = 135)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>55.19 ± 0.86²</td>
<td>48.87 ± 1.26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Males/females</td>
<td>149/139</td>
<td>69/66</td>
<td>0.14</td>
</tr>
<tr>
<td>Smoking habit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current [n (%)]</td>
<td>43 (18)</td>
<td>23 (17)</td>
<td>0.90</td>
</tr>
<tr>
<td>Former [n (%)]</td>
<td>84 (35)</td>
<td>25 (19)</td>
<td>0.0009</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.08 ± 0.33</td>
<td>24.29 ± 0.25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>32.47 ± 0.53</td>
<td>26.36 ± 0.63</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>98.12 ± 0.83</td>
<td>85.72 ± 0.90</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.93 ± 0.01</td>
<td>0.86 ± 0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>146.70 ± 1.32</td>
<td>125.22 ± 1.38</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>82.07 ± 0.77</td>
<td>74.73 ± 0.79</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>7.38 ± 0.12</td>
<td>5.41 ± 0.09</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>4.72 ± 0.13</td>
<td>3.32 ± 0.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>1.27 ± 0.03</td>
<td>1.70 ± 0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>2.61 (1.70–4.5)⁴</td>
<td>1.06 (0.86–1.39)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/L)</td>
<td>5.92 ± 0.10</td>
<td>4.99 ± 0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alcohol intake (units/wk)³</td>
<td>8.0 (2.0–14.0)</td>
<td>5 (2.0–14.0)</td>
<td>0.28</td>
</tr>
</tbody>
</table>

¹ Categorical data were compared by using Fisher’s exact or chi-square test. Between-group comparisons were assessed by one-way ANOVA.
² ± SEM (all such values).
³ Median; interquartile range in parentheses (all such values).
⁴ 1 Glass of wine = 1.5 units; 16 oz (0.47 L) of beer = 2–3 units.
Serum vitamin E concentrations

The dyslipidemic patients had significantly higher serum vitamin E concentrations than did the control subjects ($P < 0.001$; Table 3). However, the ratio of vitamin E to total cholesterol did not differ significantly between these groups ($P > 0.05$). Again, the results remained the same after adjustment of the serum vitamin E data for age. There was a strong inverse correlation between serum vitamin E concentrations and HSP-70 antibody titers ($r = -0.32, P < 0.001$) in the control group.

Dietary intakes of macronutrients and micronutrients

The dyslipidemic patients had significantly higher dietary intakes of protein and total fat (both: $P < 0.05$) than did the control subjects (Table 4). No significant difference was observed in dietary intakes of carbohydrate, sugar, and energy between the patients and the control subjects (Table 4).

The dyslipidemic patients had significantly ($P < 0.05$) higher intakes of monounsaturated fat than did the control subjects (Table 4). There was no significant difference between the control subjects and the dyslipidemic patients with respect to dietary intakes of cholesterol, saturated fat, polyunsaturated fat, or antioxidants. In addition, dietary intakes of antioxidants did not differ significantly between the dyslipidemic patients and the control subjects ($P > 0.05$; Table 4). Adjustment of dietary intake data for age had no effect on the statistical significance of the results.

Multivariate analysis

For anti-HSP-60 titers, the best-fitting models explained 11% of the variation in the dyslipidemic patients and 3.5% of that in the control subjects (Table 5). The results of a similar analysis for anti-HSP-65 titers are shown in Table 6. In the dyslipidemic subjects, 7.3% of the variation in titers was attributable to dietary vitamin C, obesity, and smoking habits. In the control subjects, 4.4% of the variation in antibody titers to HSP-65 was attributed to dietary vitamin E. For anti-HSP-70 titers, the best-fitting model explained 3.7% of the variation in the dyslipidemic patients and 21% of that in the control subjects (Table 7).

DISCUSSION

This is the first reported investigation of the relation between plasma anti-HSP titers and dietary factors in humans. Our results

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**TABLE 4**

Comparison of macro- and micronutrient dietary intakes between dyslipidemic patients and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Patients ($n = 238$)</th>
<th>Control subjects ($n = 135$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteins (g)</td>
<td>87.6 ± 1.9</td>
<td>80.9 ± 2.0</td>
<td>0.02</td>
</tr>
<tr>
<td>Total fat (g)</td>
<td>76.8 ± 1.6</td>
<td>71.5 ± 2.0</td>
<td>0.04</td>
</tr>
<tr>
<td>CHO (g)</td>
<td>282.6 ± 8.6</td>
<td>276.2 ± 9.1</td>
<td>NS</td>
</tr>
<tr>
<td>Energy (MJ)</td>
<td>10.0 ± 0.3</td>
<td>10.3 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>Sugar (g)</td>
<td>145.4 ± 5.9</td>
<td>160.8 ± 7.2</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Fat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturated (g)</td>
<td>29.4 ± 0.9</td>
<td>28.2 ± 0.6</td>
<td>NS</td>
</tr>
<tr>
<td>Monounsaturated (g)</td>
<td>27.7 ± 0.6</td>
<td>25.7 ± 0.8</td>
<td>0.04</td>
</tr>
<tr>
<td>Polyunsaturated (g)</td>
<td>10.6 ± 0.2</td>
<td>10.0 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>410.0 ± 7.2</td>
<td>392.0 ± 9.0</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Antioxidants</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>6.5 ± 0.2</td>
<td>6.1 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>42.5 ± 2.9</td>
<td>48.5 ± 4.2</td>
<td>NS</td>
</tr>
<tr>
<td>Carotene (µg)</td>
<td>2145.7 ± 79.0</td>
<td>2055.2 ± 85.3</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>16.2 ± 0.5</td>
<td>15.4 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Folate (µg)</td>
<td>288.0 ± 7.8</td>
<td>268.4 ± 7.7</td>
<td>NS</td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>393.4 ± 11.7</td>
<td>387.7 ± 12.4</td>
<td>NS</td>
</tr>
<tr>
<td>Phosphorus (mg)</td>
<td>1626.6 ± 38.4</td>
<td>1600.0 ± 41.8</td>
<td>NS</td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>4080 ± 848</td>
<td>4180 ± 1508</td>
<td>NS</td>
</tr>
</tbody>
</table>

---

**TABLE 5**

Multifactorial analysis of anti-heat shock protein 60 (absorbance units)

<table>
<thead>
<tr>
<th>Group and confounders</th>
<th>Coefficient</th>
<th>$R^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dyslipidemic patients ($n = 238$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dietary vitamin C</td>
<td>0.0000003</td>
<td>4.1</td>
<td>0.03</td>
</tr>
<tr>
<td>Sex</td>
<td>0.0019</td>
<td>2.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
<td>0.068</td>
<td>1.3</td>
<td>0.04</td>
</tr>
<tr>
<td>Dietary vitamin E</td>
<td>-0.0102</td>
<td>1.2</td>
<td>0.04</td>
</tr>
<tr>
<td>Total fat intake</td>
<td>0.0017</td>
<td>1.8</td>
<td>0.03</td>
</tr>
<tr>
<td>Total effect</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control subjects ($n = 135$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dietary monounsaturated fat</td>
<td>-0.004</td>
<td>2.2</td>
<td>0.04</td>
</tr>
<tr>
<td>Dietary carotene</td>
<td>0.00002</td>
<td>1.3</td>
<td>0.12</td>
</tr>
<tr>
<td>Total effect</td>
<td>3.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**TABLE 6**

Multifactorial analysis of anti-heat shock protein 65 (absorbance units)

<table>
<thead>
<tr>
<th>Group and confounders</th>
<th>Coefficient</th>
<th>$R^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dyslipidemic patients ($n = 238$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dietary vitamin C</td>
<td>0.000001</td>
<td>5.3</td>
<td>0.002</td>
</tr>
<tr>
<td>Obesity</td>
<td>0.05493</td>
<td>1.2</td>
<td>0.08</td>
</tr>
<tr>
<td>Smoking habit</td>
<td>-0.05149</td>
<td>0.8</td>
<td>0.12</td>
</tr>
<tr>
<td>Total effect</td>
<td>7.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control subjects ($n = 135$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dietary vitamin E</td>
<td>-0.032</td>
<td>4.4</td>
<td>0.02</td>
</tr>
</tbody>
</table>

---

**TABLE 7**

Multifactorial analysis of anti-heat shock protein 70 (absorbance units)

<table>
<thead>
<tr>
<th>Group and confounders</th>
<th>Coefficient</th>
<th>$R^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dyslipidemic patients ($n = 238$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolic syndrome</td>
<td>0.0646</td>
<td>1.9</td>
<td>0.04</td>
</tr>
<tr>
<td>Accumulation features of metabolic syndrome</td>
<td>0.0356</td>
<td>1.8</td>
<td>0.04</td>
</tr>
<tr>
<td>Total effect</td>
<td>3.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control subjects ($n = 135$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum vitamin E</td>
<td>-0.00001</td>
<td>10.3</td>
<td>0.001</td>
</tr>
<tr>
<td>Dietary carotene</td>
<td>-0.00003</td>
<td>3.8</td>
<td>0.02</td>
</tr>
<tr>
<td>Dietary cholesterol</td>
<td>0.00032</td>
<td>3.6</td>
<td>0.05</td>
</tr>
<tr>
<td>Age</td>
<td>-0.0019</td>
<td>3.3</td>
<td>0.03</td>
</tr>
<tr>
<td>Total effect</td>
<td>21.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

1 Stepwise regression was used.
Heat shock protein antibody titers and coronary artery disease risk factors

Pockley et al (36) reported that HSP-65 and -70 antibody titers were elevated in subjects with hypertension, whereas anti-HSP-60 and -70 antigen concentrations and HSP-60 antibody titers were similar to those in normotensive controls. Titers of HSP-60, -65, and -70 antibody are positively related to the risk of vascular disease and to cardiovascular disease endpoints (1). Although high immunoglobulin A—class HSP-60 antibody titers were predictive of CAD risk, the effects were reported to be modest in the absence of other classic risk factors (37). High titers of human HSP-70 are associated with a low CAD risk, probably through HSP-70’s protective effects on the cellular response to stress (38). Kocsis et al (39) reported that anti-HSP-70 titers are not elevated in subjects with severe coronary atherosclerosis, whereas anti-HSP-60 and -65 titers were significantly higher in these patients, but there was no association between titers of HSP-70 antibody and either anti-HSP-60 or -65 titers.

Heat shock protein antibody titers and dietary intake of antioxidants

Vitamin E is a potent lipid-soluble antioxidant, and its dietary supplementation has been reported to prevent oxidative injury (40). Vitamin E depletion in rats caused a significant increase in HSP-32 and -70 expression by alveolar and liver cells, which returned almost to normal after vitamin resupplementation (41). The possible modulation of the heat shock signal transduction pathway by vitamin E was previously reported in human skin fibroblasts (42). In the current study, we found an inverse relation between dietary vitamin E intake and anti-HSP-60 titers in patients and anti-HSP-65 titers in the control group. This association may be related to the redox mechanisms affected by vitamin E.

Vitamin C is an important water-soluble antioxidant that is potentially beneficial in reducing oxidative tissue damage by chemical reduction of oxidant species (43). Epidemiologic data suggest that a high intake of vitamin C may protect against oxidative damage in vivo (23, 44). A vitamin C—induced increase in baseline expression of HSP-60 and HSP-70 was previously reported, but there is controversy about the mechanism of this increase, and it has been suggested that vitamin C may exert prooxidant effects in some situations (23). In the current study, the positive association between dietary vitamin C intake and anti-HSP-60 and -65 in dyslipidemic patients may be related to an increased expression of HSPs associated with a prooxidant effect of vitamin C.

Heat shock protein antibody titers and dietary fat intake

Several studies showed that both saturated and unsaturated fatty acids can affect immune responses (17). Romano et al (15) showed that a diet rich in saturated fatty acids induces the expression of HSP-25, -60, and -70 in mice splenic lymphocytes, and we showed that serum anti-HSP-65 titers rise in rabbits fed a high-cholesterol diet (24). In the current study, we found positive associations between anti-HSP titers and dietary total fat, saturated fat, and cholesterol. These associations may be due to a combination of increased expression of the HSPs and an enhanced immune response, both of which are associated with a high-saturated-fat diet. No significant relations were found between anti-HSP titers and unsaturated fat in the current study.

Conclusions

We showed that antibody titers to HSP-60, 65, and -70 are significantly higher in patients with classical coronary risk factors than in those without such factors and are associated with dietary factors. A limitation of our study is its cross-sectional design. It is not possible to be certain that the associations between dietary constituents and HSP antibody titers are due to direct causation. In addition, our study group was a small heterogeneous sample of dyslipidemic patients. It would be important to confirm these findings in a larger sample and perhaps to examine in greater depth the relation in nondyslipidemic patients with metabolic syndrome. Nevertheless, our findings are consistent with previous studies in which a direct effect of antioxidants and fatty acids on HSP antibody titers was shown.

The contributions of authors to this paper are as follows: design of the experiment (MGM, GAF, and SAN); recruitment of subjects (MGM, GAF, CL, and TW); measurement of high-sensitivity C-reactive protein (MGM and NV); measurement of antibody titers to heat shock proteins (MGM, DFL, and GAF); measurement of vitamin E (MGM and BJS); analysis of food-frequency questionnaires (MGM and SAN); statistical analysis (MGM, SAN, and GAF); and manuscript preparation (all). MGM is a scholar of the Iranian Government. DFL was supported by the British Heart Foundation and the University of Surrey. None of the authors had any conflict of interest.

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TAS2R38 (phenylthiocarbamide) haplotypes, coronary heart disease traits, and eating behavior in the British Women’s Heart and Health Study1–4

Nic J Timpson, Mikkel Christensen, Debbie A Lawlor, Tom R Gaunt, Ian N Day, Shah Ebrahim, and George Davey Smith

ABSTRACT

Background: Variation in the perception of bitter tastes has been associated with eating behavior, body composition, and cardiovascular disease. Recent observations have implicated 2 common haplotypes of TAS2R38 in the determination of bitter compound–tasting ability.

Objective: The objectives of the study were to examine, in the British Women’s Heart and Health Study cohort, any association between TAS2R38 haplotypes, coronary heart disease (CHD), CHD risk factors, and eating behavior.

Design: We conducted a cross-sectional study of relations between TAS2R38 haplotypes and CHD, CHD risk factors, and eating behavior in 3383 women from 23 British towns.

Results: Genotyping at P49A and V262A in TAS2R38 (rs713598 and rs1726866) allowed construction of all 4 possible haplotypes. The 2 most common haplotypes corresponded with previously identified haplotypes related to bitter compound–tasting ability. No substantial evidence of association was found between these haplotypes and CHD (odds ratio for taste-defining haplotypes: 0.97; 95% CI: 0.78, 1.2), body mass index (difference between means of taste-defining haplotypes: –0.084; 95% CI: –0.45, 0.29), or a series of physiologic and dietary characteristics. A marginally lower risk of diabetes was observed among those with the nontaster haplotype than among those with the taster haplotype (odds ratio: 0.69; 95% CI: 0.48, 1.00).

Conclusion: TAS2R38 status was not an important determinant of CHD, related risk factors, or eating behavior in the British Women’s Heart and Health Study sample.

KEY WORDS Taste, TAS2R38, haplotype, behavior, coronary heart disease, CHD

INTRODUCTION

Bitter-taste perception is a classically variable trait both within and between human populations (1). The prevalence of “taste blindness” (ie, a lack of sensitivity to or an inability to taste bitter chemicals) ranges from ≈3% in West Africa to 6–23% in China and 40% in India; 30% of the white North American populations has taste blindness (1, 2).

Investigators recently reported that haplotypic variation in the region of chromosome 7 containing the TAS2R38 taste receptor gene shows a strong association with bitter compound–tasting ability (3). As such, variation across TAS2R38 is currently recognized as highly indicative of individual bitter compound–tasting ability (3).

An indirect effect of the ability to taste bitter compounds has been observed in the consequent avoidance of a host of bitter-tasting substances (4, 5). Many of these substances have antioxidant properties, and thus the tasting ability that leads to their avoidance has been implicated in the etiology of common disorders (6, 7). Although this association provides a hypothetical explanation for the involvement of bitter compound–tasting ability in disease, there is considerable debate about the role of antioxidant vitamins in the etiology of common conditions, particularly CHD (8).

However, observations suggested that lipid pathways involved in the etiology of CHD may be affected by tasting ability (9). Although it was not consistently reported (10), a preference for sweet and high-fat food was observed to decrease with increasing perception of bitter taste (11-13), and further research highlighted relations between bitter compound–tasting ability and body mass index (BMI; in kg/m²), adiposity levels, and risk factors for cardiovascular disease (14, 15). Given such evidence, the availability of genetic markers for tasting ability may offer insight into individuals’ predisposition to CHD or CHD risk traits and an opportunity to use novel approaches to the dissection of disease-environment interactions.

1 From the Department of Social Medicine, University of Bristol, United Kingdom (NJT, DAL, IND, SE, and GDS), and the Human Genetics Division, School of Medicine, University of Southampton, United Kingdom (MC, TRG, and IND).

2 The views expressed in this publication are those of the authors and not necessarily those of any of the funding bodies. The funding bodies have had no influence over the scientific work or its publication.

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4 Reprints not available. Address correspondence to NJ Timpson, Department of Social Medicine, University of Bristol, Canynge Hall, Whiteladies Road, Bristol, BS8 2PR UK. E-mail: n.j.timpson@bris.ac.uk.

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Whereas conventional observational studies have suggested that dietary components are associated with CHD risk (16-24), such associations are prone to confounding factors and reverse causality (8). Mendelian randomization has been suggested as a potential method for overcoming these difficulties (25, 26). In Mendelian randomization, genetic markers predicting intermediate trait status (inherently randomized with respect to environmental factors) allow unconfounded and directed assessment of the role that lifetime exposure to environmental risk factors may play in the development of health outcomes. It was therefore hypothesized that, if genetic factors influencing dietary preference have an effect on the health status of individual persons, genetically predicted taster status could then be used to create unconfounded tests that are capable of assigning the direction of relation (ie, the ability to avoid reverse causation) of the association between eating behavior and CHD traits.

SUBJECTS AND METHODS

The British Women’s Heart and Health Study

The British Women’s Heart and Health Study (BWHHS) was designed to examine the causes and consequences of heart disease in women. Full details of this study were reported previously (27, 28). Between 1999 and 2001, 4286 women aged 60 to 79 years, randomly selected from 23 British towns, were interviewed and examined, and they completed medical questionnaires.

Methods used at baseline assessment were described previously (27, 28). Briefly, blood samples were taken after a minimum 6-h fast. These samples were used for assessment of insulin resistance [homeostasis model assessment (HOMA) calculated from fasting insulin and glucose concentrations] and lipids (29). Blood pressure, height and weight (used for calculating BMI), and waist and hip circumferences were measured by using standard procedures (28). Prevalent CHD was defined as a medical record of myocardial infarction [based on World Health Organization diagnostic criteria (29)] or angina, a self-report of a physician’s diagnosis of either of these conditions, or both (27). Prevalent diabetes was defined as a medical record or a self-report of a physician’s diagnosis. For the purpose of the analyses presented here, these 6 categories were collapsed into 2 categories: green vegetable avoiders (the first 3 categories of consumption frequency) and green vegetable eaters (the last 3 categories of consumption frequency). Women were also asked which type of fat they normally used for spreading. In efforts to assess preference for plain fat consumption, these groups were then also collapsed to represent those who reported the use of spreadable fat and those who reported not using spreadable fat.

Alcoholic beverage consumption also was divided into 6 categories: never, on special occasions, once or twice a month, weekends only, most days, and daily. Again, for the purpose of analyses, these 6 categories were collapsed into 2 categories: consumption of alcohol at any frequency and no consumption of alcohol.

Participants were asked for informed consent to the reviewing of their medical records and to the performance of anonymous genetic tests relating to cardiovascular disease on stored blood. Eight women declined to give consent and were not included in this study. Approval for the study protocol was obtained from local ethics committees in the 23 British towns and from the UK Multicentre Ethics Committee.

Determination of TAS2R38 genotypes

A salting-out procedure was used to extract DNA from whole blood or cell residues that had been stored in tubes with K-EDTA at −80 °C for 1–2 y (30). Quantitation was done with the use of the PicoGreen assay (Molecular Probes, Eugene, OR), and DNA concentrations were equalized by dilutions with water. Long-term stock DNA aliquots were placed in storage, and working 96-well plates of DNA dilutions to 10 ng/µL were prepared. Degenerate oligo primer (DOP) amplifications were made from dilution plates to conserve stock DNA, and 384-well polymerase chain reactions (PCRs) were performed from DOP-DNA representing 1 ng of original genomic DNA. The DOP protocol was a modified version of the method used by Cheung and Nelson (31) to minimize the loss of representation of GC-rich genomic regions.

The TAS2R38 (P49A and V262A) genotypes were determined by using the melting of fluorescence-labeled oligonucleotide from matched or mismatched target, which was monitored in a 384-well Odyssey post-PCR thermal ramp (Idaho Technology, Salt Lake City, UT). Detection used reduction of dabcyI quenching of fluorescence during a thermal ramping. Asymmetric PCR was performed on 2 µL of dried DOP-amplified template in 384-well white PCR plates (Abb Gene, Epson, United Kingdom) on a MJ Research PTC-225 DNA Engine Tetrad (Genetic Research Instrumentation Ltd, Braintree, United Kingdom). The TAS2R38 P49A variant was amplified with the use of the primers 5’-GCCAGAGTTGCTTGTTTGCA-3’ at 100 nmol/L and 5’-CTGTGAGTTGTTCAGTGTTTTCAGCCA-3’ at 500 nmol/L. A fluorescein isothiocyanate (FITC)-labeled probe (with 3’ phosphate) of 5’-FITC-CTACGCTCCCTGCTCT-

PHOS-3’ matching the wild-type sequence and an adjacent dabcyI quencher, 5’-GAGACACAGACGACACACATCAGTCT-

DABCYL-3’, were included at 200 nmol/L in the PCR for the Odyssey melting assay. The TAS2R38 V262A variant was amplified with the use of the primers 5’-TGCCAGAGGGAC-

AGCTGCCATT-3’ at 100 nmol/L and 5’-TGAGAAGGCA-

TGGACGATGAAG-3’ at 500 nmol/L. An FITC-labeled probe (with 3’ phosphate) of 5’-FITC- GAAGGCAAGAGAGT-

PHOS-3’ matching the wild-type sequence and an adjacent dabcyI quencher, 5’-GCCACAGACATCGAGGGGAC-

GAG-DABCYL-3’, were included at 200 nmol/L in the PCR for the Odyssey melting assay.

The 5-µL portion of PCR mix also contained 1× PCR buffer, 0.2 mmol deoxynucleotide triphosphate/L, 1.5 mmol MgCl₂/L, and 0.1 unit Taq DNA polymerase (all: Promega, Southampton, United Kingdom). PCR cycling conditions were 94 °C for 2 min and then 99 cycles of 94 °C for 30 s, 62 °C for 30 s and, 72 °C for 30 s, which were followed by a cycle at 72 °C for 2 min. Samples were overlaid with 5 µL Chill-Out wax (Genetic Research Instrumentation) to prevent evaporation during analysis. After PCR amplification, samples were melted from 45 °C to 75 °C in the 384-well Odyssey, LIGHTTYPER software [Gaunt TR, Hinks LJ, Christensen MB, Kiesling M, Day INM. Uses of the
LightTyper in human genotype analysis: SNPs, microhaplotypes and large insertion/deletions. Biochemica (in press) was used to analyze the change in fluorescence during melting and to group melting profiles into genotype groups. These values were then verified manually by using in-house software.

### Haplotype construction

Haplotypes and predicted taster status identified in relation to previous literature (ie, a person’s status as either a taster or a nontaster of bitter compounds) were summarized in Table 1. The haplotype names PAV and AVI refer to recognized taster and nontaster states, respectively (3), and are specifically derived from their protein coding sequences. In the context of this study, haplotypes AVI and PAV have been renamed AV and PA, respectively. A person was therefore designated a nontaster if he or she carried 2 copies of AV and a taster if he or she carried ≥1 copy of PA.

Previous results showed the existence of the variant site TAS2R38 V296I (34 bases from TAS2R38 V262A) in total linkage disequilibrium with TAS2R38 V262A in a European population (3). In light of this, it was felt appropriate that the genotyping of TAS2R38 P49A and TAS2R38 V296I alone would allow the effective tagging of common haplotypes in this region.

When genotype data were collected, haplotypes were constructed by using the PHASE software program (version 2.02; Internet: http://www.stat.washington.edu/stephens/phase.html; also: 32, 33). This software employs a Bayesian method for the reconstruction of chromosomal phase by using genotype data, and it generates counts and frequencies of observed haplotypes. In cases with one missing genotype, PHASE software was used to infer the haplotypic value. The underlying method in this approach is a Markov Chain-Monte Carlo procedure in which the probability of preceding observations (in this case, unambiguous phase information) allows population genetic inference about unresolved haplotypic phase.

Our prior hypothesis, which was based on the report of Kim et al (3), was that CHD trait and eating behavior phenotypes would differ according to taster status as defined by haplotypic complement (homozygous for AV = nontaster; heterozygous or homozygous for PA = taster; other = excluded). This model formed the basis of test 1, in which 15 phenotypes, some interrelated, were coded for analysis. Subsequent post hoc analysis (test 2) was a more extreme comparison between those subjects who were homozygous for AV (conventionally recognized nontasters) and those who were homozygous only for PA (a select proportion of those haplotypically defined as tasters).

#### Statistical analysis

On the basis of observed total allele combinations, genotype distributions for single-nucleotide polymorphisms were examined for consistency with Hardy-Weinberg equilibrium by using chi-square tests. Simple two-sample t tests with unequal variances were used to compare mean values of continuous variables by haplotype-derived category. Chi-square tests were also used to compare proportions of dichotomous variables by haplotypic category. In the case of dichotomous variables, logistic regression was used to estimate odds ratios (ORs) for outcomes in question. Given their skewed distribution, HOMA scores and triacylglycerol concentrations were log transformed to approximate a normal distribution for analyses. As such, the analyses used transformed data, but the data presented are geometric means, 95% CIs, and the proportionate difference between these haplotype-derived taster groups (ie, tasters and nontasters). All statistical analyses were performed with STATA data analysis software (version 8; Stata Corp, College Station, TX).

#### RESULTS

### The British Women’s Heart and Health Study

Of the 4286 BWHHS participants at baseline, 441 had either no blood samples taken (because venipuncture did not succeed) or had insufficient blood taken for adequate samples to be stored. An additional 37 women refused consent for the use of stored

### TABLE 1

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Current study label</th>
<th>P49A Amino acid</th>
<th>V262A Amino acid</th>
<th>I296V Amino acid</th>
<th>Taster status</th>
</tr>
</thead>
<tbody>
<tr>
<td>AV[I] AV</td>
<td>G</td>
<td>T</td>
<td>[A]</td>
<td>Nontaster if homozygote</td>
<td></td>
</tr>
<tr>
<td>AA[V] AA</td>
<td>G</td>
<td>C</td>
<td>[G]</td>
<td>Small effects on nontaster status when heterozygote AV/AAV</td>
<td></td>
</tr>
<tr>
<td>PV[I] PV</td>
<td>C</td>
<td>T</td>
<td>[A]</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>PA[V] PA</td>
<td>C</td>
<td>C</td>
<td>[G]</td>
<td>Taster if homozygote or heterozyzote</td>
<td></td>
</tr>
</tbody>
</table>

- Bracketed letters indicate exclusion of that variant in the current study. Haplotype labels are derived from amino acid coding patterns.
- Derived from Kim et al (8).

### TABLE 2

Allele frequencies at variant loci TAS2R38 P49A and TAS2R38 V262A observed in the British Women’s Heart and Health Study sample

<table>
<thead>
<tr>
<th>Positions</th>
<th>Nucleotide</th>
<th>Amino acid</th>
<th>Allele</th>
<th>Amino acid</th>
<th>Frequency in the current study</th>
<th>P ( ^{1} )</th>
<th>Frequency in Kim et al (8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>145</td>
<td>49</td>
<td>G</td>
<td>Ala</td>
<td>0.60</td>
<td>0.3</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>785</td>
<td>262</td>
<td>T</td>
<td>Val</td>
<td>0.56</td>
<td>0.9</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>0.44</td>
<td></td>
<td>0.38</td>
<td></td>
</tr>
</tbody>
</table>

- Chi-square test for Hardy-Weinberg equilibrium.
blood. Of the remaining 3808 participants, DNA from 255 was not available for assay at the time of genotyping. It is notable that these participants did not differ in any systematic way from the women for whom DNA was available. A total of 3383 samples qualified for analyses because successful genotypes had been scored for at least P49A and V262A at TAS2R3. In the BWHHS sample, the minor allele frequencies for P49A and V262A were found to be 0.4 and 0.44, respectively (Table 2). These variants were in Hardy-Weinberg equilibrium, and the frequencies observed approximately match those reported by Kim et al (3).

Haplotype construction identified 3 main haplotype combinations corresponding with published work (3). PHASE successfully replaced missing genotypes (n = 399 over both single-nucleotide polymorphisms) with a corresponding ≥85% probability of accuracy in any cases. The AV haplotype [equivalent to the common AVI (nontaster) haplotype observed by Kim et al (3) and Wooding et al (34)] accounted for 56% of observed haplotypes, whereas the PA haplotype [equivalent to the common PAV (taster) haplotype also observed in the same studies] accounted for ≈40% of all haplotypes (Table 3).

The rarer haplotype, AA, has been recognized previously (3) and is here termed AAV, as a novel recombinant at low frequency. In the BWHHS sample, this haplotype was observed at a frequency of 4%. Approximately 0.1% of samples were inferred by PHASE to carry the previously unrecognized haplotype PV, but these inferences and primary data have not been followed up.

**TABLE 3**

Haplotype construction and comparative frequencies for the TAS2R38 locus in the British Women’s Heart and Health Study (BWHHS) sample and as reported by Kim et al

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Kim et al (3)</th>
<th>P49A</th>
<th>V262A</th>
<th>Frequency in Europeans (3)</th>
<th>Observed frequency in BWHHS</th>
<th>SE</th>
<th>Taster status</th>
</tr>
</thead>
<tbody>
<tr>
<td>AV</td>
<td>AV[I]</td>
<td>G</td>
<td>T</td>
<td>0.47(^\d)</td>
<td>0.56(^\d)</td>
<td>0.0006</td>
<td>Homozygote nontaster</td>
</tr>
<tr>
<td>AA</td>
<td>AA[V]</td>
<td>G</td>
<td>C</td>
<td>0.03</td>
<td>0.04(^\d)</td>
<td>0.0009</td>
<td>—</td>
</tr>
<tr>
<td>PA</td>
<td>PA[V]</td>
<td>C</td>
<td>C</td>
<td>0.49(^\d)</td>
<td>0.40(^\d)</td>
<td>0.0006</td>
<td>Homozygote or heterozygote taster</td>
</tr>
</tbody>
</table>

\(^\d\) The novel PV haplotype was observed. A new recombinant was detected as a result of the BWHHS sample size. The novel haplotype PV was observed at a frequency of 0.00053 [SE 0.0001; n = 3] and was included in analyses where appropriate.

Association between TAS2R38 haplotypes and CHD traits and eating behavior

There was no evidence of association between predicted taster status and CHD; BMI; waist-hip ratio; serum HDL, LDL, or triacylglycerol concentration; or HOMA score (Table 4; Table 5; Table 6; Table 7). There was a marginal tendency for the underrepresentation of diabetes among nontasters (test 1, OR: 0.69; 95% CI: 0.48, 1; P = 0.05; test 2, OR: 0.55; 95% CI: 0.35, 0.87; P = 0.01).

Dietary traits hypothesized to be associated with predicted bitter compound–tasting ability also failed to show significant segregation with TAS2R38 haplotypes either in test 1 or test 2. The avoidance of fats, alcohol, and green vegetables all yielded null results with respect to potential association with TAS2R38 variation. It is notable that, in the case of green vegetables, more extreme groupings of avoided vegetables were analyzed (data not shown). These groupings did not show marked differences in proportion by TAS2R38 haplotype (although these analyses were imprecise as a result of small numbers in extreme cells).

**TABLE 4**

Relation between coronary heart disease (CHD), CHD risk variables, eating behavior, and TAS2R38 haplotypes in the British Women’s Heart and Health Study

<table>
<thead>
<tr>
<th>Dichotomous traits</th>
<th>Nontasters (n = 1065)</th>
<th>Tasters (n = 2147)</th>
<th>P(^2)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHD</td>
<td>15.96% (13.88–18.29) [170](^4)</td>
<td>16.35% (14.84–17.97) [351]</td>
<td>0.8</td>
<td>0.97 (0.8, 1.19)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3.85% (2.85–4.85) [41]</td>
<td>5.45% (4.57–6.42) [117]</td>
<td>0.05</td>
<td>0.69 (0.48, 1)</td>
</tr>
<tr>
<td>Avoid green vegetables</td>
<td>19.56% (17.17–22.2) [187]</td>
<td>18.29% (16.62–20.29) [351]</td>
<td>0.4</td>
<td>1.09 (0.89, 1.32)</td>
</tr>
<tr>
<td>Avoid fats</td>
<td>3.30% (2.36–4.61) [33]</td>
<td>3.52% (2.79–4.42) [70]</td>
<td>0.8</td>
<td>0.94 (0.61, 1.43)</td>
</tr>
<tr>
<td>Avoid alcohol</td>
<td>14.95% (12.87–17.35) [144]</td>
<td>16.71% (15.12–18.42) [329]</td>
<td>0.2</td>
<td>0.88 (0.79, 1.08)</td>
</tr>
<tr>
<td>Never smoked</td>
<td>54.46% (51.46–57.43) [580]</td>
<td>56.74 (54.63–58.82) [1217]</td>
<td>0.2</td>
<td>0.91 (0.79, 1.06)</td>
</tr>
</tbody>
</table>

\(^\d\) Nontaster and taster haplotypes are defined as homozygous for AV = nontaster, heterozygous or homozygous for PA = taster, other = excluded (test 1).

\(^2\) Chi-square test.

\(^4\) Range in parentheses (all such values); n positive in brackets.
TABLE 5
Relation between continuous coronary heart disease (CHD) risk variables and TAS2R38 haplotypes in the British Women’s Heart and Health Study

<table>
<thead>
<tr>
<th>Continuous traits</th>
<th>Nontasters (n = 1065)</th>
<th>Tasters (n = 2147)</th>
<th>Difference between means (95% CI)</th>
<th>p^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>27.55 ± 4.96 [1057] \textsuperscript{a}</td>
<td>27.63 ± 5.06 [2124]</td>
<td>−0.084 (−0.45, 0.29)</td>
<td>0.7</td>
</tr>
<tr>
<td>WHR</td>
<td>0.816 ± 0.066 [53]</td>
<td>0.82 ± 0.069 [2117]</td>
<td>−0.0037 (−0.0037, 0.0013)</td>
<td>0.2</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>158.47 ± 6.05 [1057]</td>
<td>158.80 ± 6.03 [2125]</td>
<td>−0.33 (−0.77, 0.12)</td>
<td>0.1</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>69.19 ± 13.08 [1059]</td>
<td>69.62 ± 13.15 [2128]</td>
<td>−0.43 (−1.4, 0.54)</td>
<td>0.4</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.66 ± 0.43 [1055]</td>
<td>1.66 ± 0.46 [2125]</td>
<td>0.0011 (−0.032, 0.034)</td>
<td>0.9</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>4.19 ± 1.06 [1030]</td>
<td>4.13 ± 1.09 [2083]</td>
<td>0.046 (−0.035, 0.13)</td>
<td>0.3</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/L) \textsuperscript{d}</td>
<td>1.66% (1.61–1.7) [1056]</td>
<td>1.68% (1.65–1.72) [2128]</td>
<td>0.98</td>
<td>0.3</td>
</tr>
<tr>
<td>HOMA \textsuperscript{d}</td>
<td>1.67% (1.61–1.74) [915]</td>
<td>1.68% (1.63–1.73) [1853]</td>
<td>1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Nontaster and taster haplotypes and defined as homozygous for AV = nontaster, heterozygous or homozygous for PA = taster, other = excluded (test 1).

\textsuperscript{b} Two-sample t test with unequal variances.

\textsuperscript{c} Geometric \bar{x}: range in parentheses. Difference given by the ratio of geometric \bar{x}.

associated risk profile of body composition and lipid profile (14). However, in this study, no strong association was observed between TAS2R38 haplotypes and either CHD traits or eating behavior.

Those haplotypically predicted to be nontasters and tasters did show a difference in the prevalence of diabetes. However, the greater prevalence of diabetes in tasters was not consistent with previously reported dietary preferences of tasters and nontasters, which would predict an association in the opposite direction (13-15). Furthermore, whereas some of the 15 variables explored which would predict an association in the opposite direction as those observed elsewhere (27). As such, it seems unlikely that errors in the determination of genotypes or the measurement of phenotypes are a likely explanation for our observations.

Failure to detect the influence of TAS2R38 haplotypes on eating behavior may reflect limitations in the assessment of bitter compound–tasting ability. The standard dietary questionnaire responses represent a broad analysis of dietary preferences and are far from being a fully qualitative and quantitative representation of exact dietary intake. Results from the BWHHS questionnaire relating to select dietary intake measures and vitamin C consumption showed similarities with those of the European Prospective Investigation into Cancer and Nutrition Study (35) (comparisons unpublished), but specific variants such as those of TAS2R38 may mark dietary patterns that are not evident in general questionnaires but that require more a specific approach to analysis in any future study. Further investigation would be aided by the direct testing of bitter compound–tasting ability through the use of more precise physiologic methods (36, 37).

Second, the association between TAS2R38 haplotypes and bitter compound–tasting ability may not be straightforward (Figure 1A). TAS2R38 is only one of many genetic determinants likely to be involved in the pathways determining taste perception. The observation that variation at this locus correctly predicts only 80% of phenylthiocarbamide tasters (3) bears direct

TABLE 6
Relation between coronary heart disease (CHD), CHD risk variables, eating behavior, and TAS2R38 haplotypes in the British Women’s Heart and Health Study

<table>
<thead>
<tr>
<th>Dichotomous traits</th>
<th>Nontasters (n = 1065)</th>
<th>Tasters (n = 549)</th>
<th>p^2</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHD</td>
<td>15.96% (13.88–18.29) [170] \textsuperscript{a}</td>
<td>14.39% (11.69–17.59) [79]</td>
<td>0.4</td>
<td>1.13 (0.85, 1.51)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3.85% (2.85–5.19) [41]</td>
<td>6.74 (4.92–9.17) [37]</td>
<td>0.01</td>
<td>0.55 (0.35, 0.87)</td>
</tr>
<tr>
<td>Avoid green vegetables</td>
<td>19.56% (17.17–22.2) [187]</td>
<td>17.6% (14.32–20.98) [87]</td>
<td>0.3</td>
<td>1.15 (0.87, 1.53)</td>
</tr>
<tr>
<td>Avoid fats</td>
<td>3.3% (2.35–4.61) [33]</td>
<td>3.1% (1.9–4.99) [16]</td>
<td>0.8</td>
<td>1.07 (0.58, 1.96)</td>
</tr>
<tr>
<td>Avoid alcohol</td>
<td>14.95% (12.84–17.35) [144]</td>
<td>15.58 (12.68–19.01) [79]</td>
<td>0.8</td>
<td>0.95 (0.71, 1.28)</td>
</tr>
<tr>
<td>Never smoked</td>
<td>54.46% (51.45–57.43) [251]</td>
<td>56.75 (52.56–60.85) [311]</td>
<td>0.4</td>
<td>0.91 (0.74, 1.12)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Nontaster and taster haplotypes are defined as homozygous for AV = nontaster, heterozygous or homozygous for PA = taster, other = excluded (test 2).
The effects of dietary composition on CHD and CHD traits) is that genetically predicted bitter compound–tasting ability to assess the relation between tasting ability and CHD and CHD risk factors. The major implication of this (beyond the suggestion not to use questionables (Figure 1C; 10). Given reliable phenotypic vari-
ables and accurate genetic data, our results indicate a lack of questionable (Figure 1C; 10). Given reliable phenotypic vari-

tichromosomes 5, 7, and 12. Tasting ability is also likely to be influ-
enced by many other sensory and proprioceptive pathways, and the probable result is that no single genetic marker has a great effect. In particular, these other pathways are likely to include olfactory contributions to food preference, although digestive and cognitive factors may also complicate the overall system and modify the ability to perceive bitter taste.

Third, under the assumption that more accurately assessed bitter compound–tasting ability is associated with TAS2R38 haplo-
types (3), the postulated relation between bitter compound–tasting ability, dietary preference, and health outcome becomes questionable (Figure 1C; 10). Given reliable phenotypic variables and accurate genetic data, our results indicate a lack of relation between tasting ability and CHD and CHD risk factors. The major implication of this (beyond the suggestion not to use genetically predicted bitter compound–tasting ability to assess the effects of dietary composition on CHD and CHD traits) is that the inherent ability to detect bitter compounds is not likely to be an important intermediate phenotype and hence a predisposing factor for CHD.

Moreover, there was a surprising lack of association between taster status and the consumption of green vegetables. Sensitivity to the bitter flavors of cruciferous vegetables is widely recognized as a common phenotype in those able to taste bitter compounds. Despite having direct questions relating to the frequency of consumption of green vegetables, we failed to observe any association with this trait. Given the lack of association with other traits, this finding may well be due to the explanations offered above, although the possibility of effect masking through sociocultural characteristics of the sample population remains. For example, older women raised in times of austerity might be expected to have less-marked food preferences. Furthermore, “debittering” through cooking or the addition of salt, sugar, or fat may effectively reduce the effect of many substances said to elicit a bitter taste response (38).

This investigation of a large, population-based sample of women has not provided support for work associating taster status with the etiology of CHD, CHD-related risk factors, or eating behavior (13-15). This lack of association between TAS2R38 gene variation and either phenotypic outcome or intermediate variable did not permit us to test potential unconfounded relations between eating behavior, CHD, and CHD risk factors by using the Mendelian randomization model (25, 26). Furthermore, larger-scale analyses in different, reasonably sized samples are required if the TAS2R38 locus is to be confirmed as having common involvement with the tasting ability and CHD risk factors. The combination of this approach with the gathering of more complete genomewide taste and olfactory receptor diversity data, and the taking of direct measures of taste perception would allow the assessment of the genetic foundations and implications of tasting ability.

We thank Carol Bedford, Alison Emerton, Nicola Frecknall, Karen Jones, Rita Patel, Mark Taylor, and Katherine Wornell for collecting and entering data; all of the general practitioners and their staff members who supported the data collection; and the women who participated in the study. The British Women’s Heart & Health Study is codirected by Shah Ebrahim, Peter Whincup, and Goya Wannamethe.

All authors developed the study aim and design. IND, together with SE and DAL, obtained funding for the DNA bank and genotyping. Genotyping was done by MC, and DNA bank and data management was done by TRG. NJT

relation to this and is likely a testament to the existence of numerous members of the TAS2R gene family clustered on chromosomes 5, 7, and 12. Tasting ability is also likely to be influenced by many other sensory and proprioceptive pathways, and the probable result is that no single genetic marker has a great effect. In particular, these other pathways are likely to include olfactory contributions to food preference, although digestive and cognitive factors may also complicate the overall system and modify the ability to perceive bitter taste.

Third, under the assumption that more accurately assessed bitter compound–tasting ability is associated with TAS2R38 haplotypes (3), the postulated relation between bitter compound–tasting ability, dietary preference, and health outcome becomes questionable (Figure 1C; 10). Given reliable phenotypic variables and accurate genetic data, our results indicate a lack of relation between tasting ability and CHD and CHD risk factors. The major implication of this (beyond the suggestion not to use genetically predicted bitter compound–tasting ability to assess the effects of dietary composition on CHD and CHD traits) is that the inherent ability to detect bitter compounds is not likely to be an important intermediate phenotype and hence a predisposing factor for CHD.

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TABLE 7
Relation between continuous coronary heart disease (CHD) risk variables and TAS2R38 haplotypes in the British Women’s Heart and Health Study

| Continuous traits | Nontasters (n = 1065) | Tasters (n = 549) | Difference between means (95% CI) | P
|-------------------|----------------------|------------------|----------------------------------|---
| BMI               | 27.55 ± 4.96 [1057]  | 27058 ± 5.03 [544] | −0.027 (−0.54, 0.49)             | 0.9
| WHR               | 0.816 ± 0.066 [1053] | 0.822 ± 0.07 [543] | −0.006 (−0.013, 0.00079)         | 0.08
| Height (cm)       | 158.47 ± 6.05 [1057] | 158.75 ± 6.14 [545] | −0.29 (−0.91, 0.34)              | 0.4
| Weight (kg)       | 69.19 ± 13.08 [1059] | 69.48 ± 13.09 [544] | −2.89 (−1.64, 1.07)              | 0.7
| HDL (mmol/L)      | 1.66 ± 0.43 [1055]   | 1.67 ± 0.47 [545] | −0.014 (−0.06, 0.032)            | 0.6
| LDL (mmol/L)      | 4.17 ± 1.06 [1030]   | 4.07 ± 1.06 [528] | 0.11 (−0.0028, 0.22)             | 0.06
| Triglycerols (mmol/L) | 1.66 (1.61–1.71) [1056] | 1.66 (1.6–1.73) [547] | 1 | 0.9
| HOMA²             | 1.67 (1.61–1.74) [915] | 1.65 (1.57–1.75) [464] | 1.01 | 0.7

¹ Nontaster and taster haplotypes are defined as homozygous for AV = nontaster, homozygous for PA = taster, other = excluded (test 2). Mean variable values by test 2 status: 0 = homozygous for AV, 1 = homozygous for PA, WHR, waist-hip ratio; HOMA, homeostasis model assessment.

² Two-sample t test with unequal variances.

³ ± SD (all such values); n in brackets.

⁴ Geometric ś; range in parentheses. Difference given by the ratio of geometric ś.

FIGURE 1. The hypothesized relation between TAS2R38 haplotypes and health outcome (A) and the alternative possible explanations for the results observed in the current study (B and C). Diagram B represents a challenge to the existence of any relation between TAS2R38 genetic variation and differential ability to taste bitter compounds. Diagram C represents a challenge to the assumption that differential ability to taste bitter compounds will have any real effect on the dietary intake of an individual person.
undertook the initial analysis and coordinated the writing of the manuscript. All authors contributed to the final version of the manuscript. NIT and IND act as guarantors. DAL is a codirector of the British Women’s Heart & Health Study. None of the authors had a personal or financial conflict of interest.

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ABSTRACT
Background: Several small-scale clinical trials have suggested a potential beneficial effect of short-term soy consumption on blood pressure (BP). Data are scanty on long-term effects of the usual intake of soy foods on BP in general populations.

Objective: Our aim was to examine the association between usual intake of soy foods and BP.

Design: The usual intake of soy foods was assessed at baseline, and BP was measured 2–3 y after the baseline survey among 45 694 participants of the Shanghai Women’s Health Study aged 40–70 y who had no history of hypertension, diabetes, or cardiovascular disease at recruitment. Multiple regression models were used to estimate mean differences in BP associated with various intakes of soy foods.

Results: Soy protein intake was inversely associated with both systolic BP ($P$ for trend = 0.01) and diastolic BP ($P$ for trend = 0.009) after adjustment for age, body mass index, and lifestyle and other dietary factors. The adjusted mean systolic BP was 1.9 mm Hg lower (95% CI: −3.0, −0.8 mm Hg) and the diastolic BP was 0.9 mm Hg lower (−1.6, −0.2 mm Hg) in women who consumed ≥25 g soy protein/d than in women consuming <2.5 g/d. The inverse associations became stronger with increasing age ($P$ for interaction < 0.05 for both BPs). Among women >60 y old, the corresponding differences were −4.9 mm Hg (95% CI: −8.0, −1.9 mm Hg) for systolic BP and −2.2 mm Hg (95% CI: −3.8, −0.6 mm Hg) for diastolic BP.

Conclusion: Usual intake of soy foods was inversely associated with both systolic and diastolic BPs, particularly among elderly women.

KEY WORDS  Blood pressure, soy foods, women, longitudinal observation

INTRODUCTION
Because of substantial evidence that soy protein intake improves serum lipid profiles (1), the US Food and Drug Administration and the American Heart Association issued a recommendation of daily consumption of ≥25 g soy protein as a preventive measure to reduce the risk of heart disease (2, 3). Recently, the nonlipid-related effects of soy, especially its influence on vascular function, have become a focus of research (4). Soy isoflavones, an important class of phytoestrogens, have been shown to decrease in vivo oxidation (5), stimulate nitric oxide production (6, 7), improve systemic arterial compliance (8–11), and favorably affect salt and water balance (12–14), all of which suggests a protective role with respect to the development of hypertension. Soy intake has also been suggested to reduce C-reactive protein concentrations (15, 16), a marker of systemic inflammation that has been associated with incident hypertension (17). Several small-scale clinical trials have further provided evidence that soy intake may be effective in lowering blood pressure (BP), although the results are not entirely consistent (18–25). No hypotensive effect of soy supplement was found in some of the previous studies (24, 25). Most of those trials, however, investigated the effects of specific soy components supplemented at a relatively high dose for a short period of time. Data are scanty on usual dietary intake of soy foods in relation to BP in general populations. Two cross-sectional studies conducted in the United States, where soy foods are rarely consumed, examined dietary intake of soy phytoestrogen and found an inverse but statistically nonsignificant association with BP (26, 27).

In the current study, we examined the association between usual intake of soy foods and BP among the participants in the Shanghai Women’s Health Study, a large cohort study that was conducted in a population that had a wide range of soy food intake and that thus was uniquely suited for an evaluation of the health effects of soy.

SUBJECTS AND METHODS
Study population
The Shanghai Women’s Health Study, initiated in March 1997, is a population-based prospective cohort study of Chinese women aged 40–70 y who are residing in 7 urban communities of Shanghai. Of the 81 170 eligible women identified from the Shanghai Resident Registry, 2407 (3.0%) refused to participate in the study, 2073 (2.6%) were not available during the study recruitment period, and 1469 (1.8%) were not enrolled for miscellaneous other reasons such as mental disorder. The remaining 75 221 women were recruited, and they completed the baseline...
survey between 1997 and 2000, for a participation rate of 92.7%. After the exclusion of women who were found to be outside of the study’s age range at the time of interview, the final cohort of the Shanghai Women’s Health Study consisted of 74,943 women.

Written informed consent was obtained from all study participants. The study was approved by the Institutional Review Board of Vanderbilt University and all other participating institutions.

Trained retired nurses conducted the baseline survey at participants’ homes by using a structured questionnaire designed to collect information on demographic characteristics, diet and lifestyle habits, medical history, and use of medications, including antihypertensives and hormones. The prevalence of hypertension was based on self-reporting because BP was not measured at baseline. Anthropometric measurements were taken with the use of a standardized protocol. All interviews were tape-recorded and selectively checked by quality-control staff to monitor the quality of the interview data. All study participants were followed through biennial in-person interviews.

Assessment of soy food consumption

At the baseline survey, the interviewers collected information on usual dietary intake over the previous 12 mo for all the cohort members through a face-to-face interview by using a validated food-frequency questionnaire (28). The questionnaire included 11 soy food items (ie, tofu, soy milk, fried bean curd, bean curd cake, and other kinds of soy products), covering virtually all soy foods consumed in urban Shanghai. Fresh and dried soybeans were also included. For each food item, study participants were first asked to report how frequently (daily, weekly, monthly, yearly, or never) they consumed the food; this question was followed by a question on the amount of intake in the Chinese measure, lians (1 lian = 50 g). For seasonal foods (eg, fresh legumes), in-season consumption patterns were determined, and the average daily consumption over a 12-mo period was calculated by adjustment for the estimated months during which the foods were consumed. We estimated total soy food intake by tallying the soy protein content for each specific soy food on the basis of the Chinese Food Composition Table (29). We also estimated isoflavone intake by using the published data on the isoflavone content of soy foods (30).

We have published elsewhere the validation study of the food-frequency questionnaire (28). Briefly, in a random sample of 200 participants in the Shanghai Women’s Health Study, we compared the estimates of dietary intake derived from the food-frequency questionnaire with those derived from 24-h dietary recalls conducted twice a month for 12 mo. The Pearson correlation coefficient for soy food intake was 0.49. The coefficients for nutrient intakes ranged from 0.41 to 0.64.

Blood pressure measurement

The first follow-up survey was conducted 2–3 y after the baseline survey, with a response rate of 99.7%. BP was measured for 91% of the participants (n = 68,427) as part of the first follow-up survey. After the participants sat quietly for ≥5 min, trained interviewers (retired nurses) measured BP with the use of a conventional mercury sphygmomanometer according to a standard protocol (31).

Statistical analysis

For the current study, we excluded women who reported a history of hypertension (n = 16,455), diabetes (n = 3,004), coronary heart disease (n = 5068), or stroke (n = 776) or who took antihypertensive medications (n = 11,086). These exclusions were made because of concerns that dietary practice and BP could be substantially influenced by disease diagnosis and use of medications. We also excluded from this analysis users of postmenopausal hormones (n = 1409) and women who underwent hysterectomy (n = 3701), out of concern that potential hormone-related mechanisms may undercut the effects of soy or soy isoflavones. In addition, we excluded women with missing BP data (n = 46) or with an extreme total energy intake (<500 or >3500 kcal/d; n = 97). After these exclusions (not mutually exclusive), 45,694 women remained for the analysis.

We applied a multiple regression model to evaluate the association between usual dietary soy protein intake and BP. We categorized the study subjects into 5 groups according to daily soy protein intake, with cutoffs being 2.5 (x –SD), 8.8 (x), 15.1 (x + SD), and 25 g/d (the minimum amount recommended by the US Food and Drug Administration). Subjects with soy protein intake <2.5 g/d were chosen as the reference group. Mean BP differences associated with each category of soy protein intake compared with the reference group (the lowest category) and their 95% CIs were estimated by using the multiple regression models that adjusted for potential confounding variables. The variables adjusted for included age (continuous), body mass index (BMI; in kg/m²; continuous), education (5 categories), household income (4 categories), alcohol consumption (yes or no), cigarette smoking (yes or no), regular physical activity during the past 5 y (yes or no), and dietary factors (continuous), such as intakes of total energy, nonsoy protein, fruit, vegetables, and sodium from both salt and food. A linear trend test was performed by treating each ordinal score variable as a continuous variable in the model. Because many dietary factors highly correlate with each other, we used the variable inflation factor to measure the effect of multicollinearity for each variable. All dietary factors included in the models had a variable inflation factor <10. To minimize a potential influence of recent dietary change on the results, we conducted separate analyses among women who reported no significant changes in vegetable consumption during the past 5 y. We also conducted analyses stratified by age and menopausal status (menopause was defined as an absence of the menstrual period for ≥12 mo, excluding lapses related to either pregnancy or breastfeeding). Tests for interaction were performed by introducing a multiplicative interaction term into the main effect models. All tests of statistical significance were based on two-sided probability. Statistical analyses were performed with the use of SAS software (version 8.2; SAS Institute Inc, Cary, NC).

RESULTS

The mean age of the study population was 49.9 ± 8.5 y, and 38.0% were postmenopausal (Table 1). Approximately 13% of the study participants had attended college or had other higher education. Few women had ever smoked cigarettes (2.5%) or consumed alcohol regularly (2.4%). More than 30% of women reported exercising ≥1 time/wk during the past 5 y. The mean intake of soy protein was 8.8 ± 6.3 g/d. A higher intake of soy
protein was more common among older and postmenopausal women. Women with higher soy protein intake were more likely to have a higher BMI and higher educational level and were also more likely to have ever been a regular alcohol drinker or exercised regularly but less likely to be a cigarette smoker than were women with lower soy protein intake. Soy intake was also associated with intakes of total energy, fruit, vegetables, sodium, and nonsoy protein.

The mean values of systolic and diastolic BPs were 117.6 ± 16.4 and 75.6 ± 9.5 mm Hg, respectively, in this study population. Age, BMI, and sodium intake were found to be positively associated with both systolic and diastolic BPs, whereas higher education and household income, regular exercise, and higher intakes of vegetables, fruit, and nonsoy protein were associated with lower systolic and diastolic BPs.

After adjustment for age and BMI, soy protein intake was inversely associated with both systolic (P for trend = 0.002) and diastolic (P for trend = 0.007) BPs (Table 2). Further adjustment for lifestyle and other dietary factors, including intakes of sodium, total vegetables, fruit, and nonsoy protein, did not appreciably change the inverse association. The adjusted mean systolic BP was 1.9 mm Hg lower (95% CI: −3.0, −0.8 mm Hg) and the diastolic BP was 0.9 mm Hg lower (−1.6, −0.2 mm Hg) in women who consumed ≥25 g soy protein/d than in women who consumed <2.5 g/d. The effects were found to be slightly greater (≈10% increase) when analyses were confined to subjects who reported no obvious changes in their consumption of vegetables during the previous 5 y. Similar inverse associations with BP were also found for dietary intake of soy isoflavones.

In stratified analyses, a tendency for a more pronounced hypotensive effect of soy food intake was found in postmenopausal women, although interaction tests were not significant (Table 3). The hypotensive effect was substantially strengthened in elderly women. Among women aged ≥60 y, daily soy protein intake ≥25 g, as compared with the lowest intake, was associated with a decrease of 4.9 mm Hg (95% CI: −8.0, −1.9 mm Hg) in systolic BP and of 2.2 mm Hg (−3.8, −0.6 mm Hg) in diastolic BP. The test for multiplicative interaction was significant for both systolic (P = 0.008) and diastolic (P = 0.01) BP.

**DISCUSSION**

In this large, population-based longitudinal study, we found that usual intake of soy food assessed at baseline was significantly and inversely associated with both systolic and diastolic BPs measured 2–3 y later in apparently healthy women. This association was independent of important risk factors for hypertension and other dietary factors. Elderly postmenopausal women appear to benefit more from soy consumption than do premenopausal women in terms of reductions in BP. Because there is a continuum of increased cardiovascular risk across levels of BP (32), and because soy products can be readily incorporated into most diets, our findings, if confirmed by further research, would have important public health implications.

The observed inverse association between soy food intake and BP is biologically plausible. Oxidative stress and inflammation have been implicated in the development of hypertension (17, 23). Soy isoflavones have been shown to reduce both in vitro and...
in vivo oxidation (5, 34). It has also been reported that genistein (an important isoflavone) stimulates the production of nitric oxide (6, 7), a factor that is known to have potent vasodilatory and antiinflammatory effects (35). In a recent clinical trial (16), a dietary portfolio of cholesterol-lowering foods, including soy foods, significantly lowered serum lipid and C-reactive protein (a marker of systemic inflammation) concentrations, and the effect size was comparable to that achieved with the initial therapeutic dose of a first-generation statin. BP is known to rise with increasing arterial stiffness (36), which relates to aging and menopause (4), and intakes of phytoestrogens from both food and supplement sources have been inversely associated with arterial stiffness among postmenopausal women (4, 8, 18, 37). It is not surprising that we observed a more pronounced inverse

<table>
<thead>
<tr>
<th>Soy protein intake (g/d)</th>
<th>SBP, age- and BMI-adjusted</th>
<th>SBP, fully adjusted</th>
<th>DBP, age- and BMI-adjusted</th>
<th>DBP, fully adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td>All subjects (n = 45 694)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2.5 (n = 4007)</td>
<td>0.0 (reference)</td>
<td>0.0 (reference)</td>
<td>0.0 (reference)</td>
<td>0.0 (reference)</td>
</tr>
<tr>
<td>2.5–8.7 (n = 23 273)</td>
<td>−0.4 (−0.9, 0.1)</td>
<td>−0.2 (−0.7, 0.3)</td>
<td>−0.3 (−0.6, 0.0)</td>
<td>−0.2 (−0.6, 0.1)</td>
</tr>
<tr>
<td>8.8–15.0 (n = 12 859)</td>
<td>−0.7 (−1.3, −0.2)</td>
<td>−0.5 (−1.0, 0.1)</td>
<td>−0.4 (−0.7, −0.1)</td>
<td>−0.4 (−0.7, −0.1)</td>
</tr>
<tr>
<td>15.1–24.9 (n = 4560)</td>
<td>−0.5 (−1.1, 0.1)</td>
<td>−0.3 (−1.0, 0.4)</td>
<td>−0.4 (−0.8, 0.0)</td>
<td>−0.4 (−0.8, 0.0)</td>
</tr>
<tr>
<td>≥25 (n = 995)</td>
<td>−1.8 (−2.8, −0.7)</td>
<td>−1.9 (−3.0, −0.8)</td>
<td>−0.8 (−1.4, −0.1)</td>
<td>−0.9 (−1.6, −0.2)</td>
</tr>
<tr>
<td>P for trend</td>
<td>0.002</td>
<td>0.097</td>
<td>0.009</td>
<td></td>
</tr>
</tbody>
</table>

Subjects with no recent change in diet (n = 39 895)

<2.5 (n = 3596) | 0.0 (reference) | 0.0 (reference) | 0.0 (reference) | 0.0 (reference) |
2.5–8.7 (n = 20 527) | −0.4 (−0.9, 0.1) | −0.2 (−0.7, 0.3) | −0.3 (−0.6, 0.0) | −0.3 (−0.6, 0.0) |
8.8–15.0 (n = 11 132) | −0.9 (−1.4, −0.3) | −0.6 (−1.2, −0.0) | −0.5 (−0.8, −0.2) | −0.5 (−0.9, −0.1) |
15.1–24.9 (n = 3848) | −0.6 (−1.3, 0.1) | −0.5 (−1.2, 0.3) | −0.5 (−0.9, −0.1) | −0.5 (−1.0, −0.1) |
≥25 (n = 792) | −2.0 (−3.1, −0.8) | −2.1 (−3.4, −0.9) | −0.8 (−1.5, −0.1) | −1.0 (−1.7, −0.3) |
| P for trend | 0.0004 | 0.002 | 0.002 | 0.001 |

1 BP measurement was taken 2–3 y after the baseline dietary survey. SBP, systolic BP; DBP, diastolic BP.
2 Mean difference in BP associated with each category of soy protein intake compared with the reference level, estimated by the multiple regression models.
3 Adjusted for age, BMI, education, household income, alcohol consumption, cigarette smoking, regular physical activity during the last 5 y, and intakes of total calories, nonsoy protein, sodium, fruit, and total vegetables.
4 Subjects who reported no major changes in vegetable consumption during the previous 5 y.
association of soy intake with BP among older postmenopausal women, because the adverse structural changes in the vessel wall are more prominent among that group. Soy intake may also lower BP through a natriuretic effect similar to furosemide (13, 14).

Data linking soy intake with BP have been limited and inconsistent. Soy consumption is extremely low in most Western populations, and this hinders epidemiologic studies of its health effects (38). In the Framingham Offspring Study of 939 postmenopausal US women, a relatively high intake of isoflavone was found to be associated with a reduction of 2.0 mm Hg in systolic BP and a reduction of 0.7 mm Hg in diastolic BP, although the associations were not significant (27). One small cross-sectional study in Japan observed a significant inverse association of BP with soy food intake in men but, unexpectedly, not in women (39). Several randomized controlled clinical trials showed that short-term soy supplementation significantly reduced both systolic and diastolic BPs (18–23, 40), and the reductions were substantially more pronounced in subjects with mild-to-moderate hypertension than in normotensive subjects (22). In an additional analysis of 11 086 subjects taking antihypertensive medication, we also found a slightly stronger inverse association between soy intake and BP (data not shown)—a mean difference of −2.8 mm Hg (95% CI: −5.2, −0.5 mm Hg) in systolic BP and of −1.7 mm Hg (−3.0, −0.5 mm Hg) in diastolic BP—when we compared the highest with the lowest intake of soy protein. In contrast, no effects of soy on BP were reported in other trials (24, 25). Differences in characteristics of the study participants, soy components being used, and the doses and durations may partly explain the inconsistency.

To our knowledge, this is the first population-based longitudinal study on usual soy food intake and BP. The large sample size and the wide range of soy consumption in our study subjects allowed us to evaluate the effect of usual soy food intake on BP in the general population, with a study power of ≥80% (α = 0.05) to detect a difference of 0.17 mm Hg in systolic BP and a difference of 0.10 mm Hg in diastolic BP associated with each 5 g/d increase in soy protein intake. The population-based prospective study design and the extremely high response rates in both the baseline and follow-up surveys eliminated potential recall bias and minimized selection bias, 2 principal concerns in most case-control studies. The dietary questionnaire used in this study has been shown to be of good validity in measuring usual intake of important nutrients and food groups (28). Moreover, dietary data were collected before BP measurement (2–3 y before). Thus, potential errors in assessment of usual dietary intake may not be a big concern in this study. The comprehensive information collected at baseline allowed us to account for potential confounding from other dietary and nondietary factors. Furthermore, very few women in our study smoked cigarettes, drank alcoholic beverages, and used hormone replacement therapy, which substantially limited the potential confounding effects of those variables on the association of soy food intake and BP.

However, this observational study cannot definitively prove a causal effect of soy consumption on BP. Women in the different categories of soy consumption also differed in several other respects, such as other dietary factors. Although careful adjustment for these potential confounding factors did not appreciably change the results (which suggests an independent effect), we could not completely exclude the possibility of residual confounding because of unmeasured or inaccurately measured covariates. For example, information on family history of hypertension was not collected in the study. People with a family history of hypertension are likely to pursue a healthy lifestyle and dietary practice. Nevertheless, we have adjusted for a broad range of potential confounding variables, and the adjustment did not materially alter the results, which suggests that the potential residual confounding is unlikely to explain away the observed robust association between intake of soy foods and BP.

Some women may have changed their usual diets around the time of the baseline survey. We found a slightly greater decrease in BP associated with soy food intake in the analyses confined to subjects with no significant changes in vegetable intake during the past 5 y, which suggests that our results could not be explained by recent dietary changes in some cohort members. The use of BP values measured on a single occasion is another limitation. Nevertheless, a single BP reading has been shown to be a strong predictor for future cardiovascular disease events (41).

In summary, we found in this large longitudinal study that usual intake of soy foods was significantly and inversely associated with both systolic and diastolic BPs, particularly among late postmenopausal women. Although the magnitude of reduction in BP associated with daily consumption of ≥25 g soy protein in the whole cohort of healthy women may not have significant clinical relevance, the public health implications may be important, given that a small reduction in populationwide BP can lead to a substantial decrease in cardiovascular risk in the society (42, 43). These data lend further support to the recommendation to increase consumption of soy foods to promote cardiovascular health.

We thank the participants and research staff of the Shanghai Women’s Health Study for their contribution to the study. We also thank Bethanie Hull for her assistance in preparing the manuscript.

REFERENCES


QDR 4500A dual-energy X-ray absorptiometer underestimates fat mass in comparison with criterion methods in adults\textsuperscript{1–4}

Dale A Schoeller, Frances A Tylavsky, David J Baer, William C Chumlea, Carrie P Earthman, Thomas Fuerst, Tamara B Harris, Steven B Heymsfield, Mary Horlick, Timothy G Lohman, Henry C Lukaski, John Shepherd, Roger M Siervogel, and Lori G Borrud

ABSTRACT
Background: Dual-energy X-ray absorptiometry (DXA) has become one of the most frequently used methods for estimating human body composition. Although the DXA technique has been validated for the measurement of fat-free mass and fat mass, differences in calibration between instruments produced by different manufacturers, as well as between different models produced by the same manufacturer, have been reported.

Objective: The objective was to compare the calibration of the QDR 4500A against criterion methods in a large heterogeneous population.

Design: DXA-derived body-composition data were obtained from 7 studies: 6 data sets were provided by the investigators, one of which was published. The data included fat mass and fat-free mass measured with a QDR 4500A and criteria measurements of body composition from total body water by dilution at 4 centers, densitometry from 1 center, and four-compartment analysis at 2 centers.

Results: In the cohort of 1195 subjects, 602 men and 593 women aged 19–82 y with a body mass index (in kg/m\textsuperscript{2}) of 16–44, the fan-beam DXA overestimated fat-free mass (P < 0.05). A significant difference was observed in all 7 data sets, and the mean (± SE) was 5 ± 1%.

Conclusions: It is recommended that the lean soft tissue mass estimate with the fan-beam QDR 4500A be reduced by 5% and that for fat mass be increased by that same mass. This finding is particularly important because the National Health and Nutrition Examination Survey is using the QDR 4500A to assess body composition in a nationally representative sample of persons in the United States. Am J Clin Nutr 2005;81:1018–25.

KEY WORDS

Body composition, hydration, total body water

INTRODUCTION

National data gathered from examination studies over the past 30 y have shown an increase in overweight and obesity across all strata of the US population on the basis of body mass index (BMI; in kg/m\textsuperscript{2}) (1, 2). Although BMI provides an acceptable approximation of total body fat (3), its use has limits because the relation between BMI and body fat varies with age, sex, physical training, and ethnicity (4–7).

A direct measure of fat-free mass (FFM) and fat mass (FM) is, therefore, often preferred for assessing obesity. Dual-energy X-ray absorptiometry (DXA) estimates of FFM and FM have been validated and generally are reported to correlate highly with values determined with criterion methods (8–14). In addition, DXA can provide estimates of fat distribution by body region, yet is rapid and simple to perform in most subjects. The DXA information on body composition from large multiethnic studies may help to identify factors that might explain differences seen in cardiovascular disease risk factors and other markers of chronic disease, including bone mineral density (BMD) (15–18). For these reasons, the National Center for Health Statistics, Centers for Disease Control and Prevention, included DXA in the current National Health and Nutrition Examination Survey (NHANES) (19).

DXA, however, is not without limitations. Although highly correlated with criterion methods, modest systematic variation in the absolute estimates of body composition by DXA can arise from different hardware and software accommodations to several factors, including interpolations for soft tissues located over bone (8) and treatment of pixels for which a small portion is bone (20). For newer and faster fan-beam instruments, parallax error (beam magnification) due to the variation in heights between the source and detector of tissues is an additional concern (11). Because of these inaccuracies in a given DXA instrument (11), it...
is important to either cross-validate new DXA models or substantive software revisions against an established DXA instrument or an established criterion method.

The accuracy of the QDR 4500A (Hologic, Bedford, MA) is of particular interest because it is being used in the continuous NHANES. The results from NHANES will provide national estimates for body composition, which should be a valuable baseline for future surveys or smaller independent samples. Future studies are likely to use other instruments or techniques; therefore, establishing the accuracy of the fan-beam QDR 4500A is critical. Recent studies, however, have indicated that the Hologic QDR 4500A overestimates FFM and underestimates FM compared with criterion methods (12, 21, 22) and the pencil-beam QDR 2000 (11, 13).

The aim of this study was to compare the QDR 4500A measurement of FM and FFM against that of measurements made with criterion methods at multiple laboratory sites. The use of multiple laboratories ensured that individual bias from any one instrument, criterion method, or protocol would be minimized and, thus, the NHANES results should be the most reliable estimates of body composition in the US population that are possible.

### SUBJECTS AND METHODS

#### Subjects

Seven data sets containing estimates of body composition from the QDR 4500A DXA and a corresponding criterion method were provided from 6 different laboratories. A seventh data set was taken from the literature (22) (Tables 1 and 2). The combined cohort ranged in age from 19 to 82 y and in BMI from 16 to 44. During the initial review, 26 data points were dropped from the analysis, including 17 because of a 3-kg weight difference between scale weight and DXA weight, 4 because of a >6-kg difference in FFM between total body water (TBW) and DXA, 3 because they were too large to fit within the DXA detector field, and 2 because TBW accounted for <51% of FFM_{DXA}. The remaining sample included 1004 participants from the 6 data sets and 191 participants from the publication of Deurenberg-Yap et al (22). The human studies were reviewed and approved by the institutional review boards of the respective institutions, and written informed consent was obtained from the participants. All data sets were stripped of participant identifiers before being used in this data analysis.

#### TABLE 1

Subject characteristics

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age</th>
<th>Weight</th>
<th>Height</th>
<th>BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n = 0 M, 22 F)</td>
<td>65 ± 4 (59–74)</td>
<td>71 ± 14 (50–104)</td>
<td>165 ± 5 (157–174)</td>
<td>26 ± 5 (19–36)</td>
</tr>
<tr>
<td>2 (n = 139 M, 139 F)</td>
<td>75 ± 3 (70–82)</td>
<td>76 ± 16 (41–127)</td>
<td>167 ± 10 (147–192)</td>
<td>27 ± 5 (16–42)</td>
</tr>
<tr>
<td>3 (n = 12 M, 12 F)</td>
<td>47 ± 10 (32–66)</td>
<td>78 ± 20 (48–130)</td>
<td>170 ± 11 (147–188)</td>
<td>27 ± 7 (17–42)</td>
</tr>
<tr>
<td>4 (n = 68 M, 3 F)</td>
<td>43 ± 11 (19–71)</td>
<td>81 ± 13 (57–117)</td>
<td>164 ± 6 (151–181)</td>
<td>30 ± 3 (22–38)</td>
</tr>
<tr>
<td>5 (n = 30 M, 28 F)</td>
<td>74 ± 2 (70–79)</td>
<td>76 ± 15 (38–111)</td>
<td>167 ± 9 (148–187)</td>
<td>27 ± 5 (17–40)</td>
</tr>
<tr>
<td>6 (n = 206 M, 245 F)</td>
<td>44 ± 15 (19–79)</td>
<td>78 ± 17 (44–125)</td>
<td>172 ± 10 (148–197)</td>
<td>26 ± 5 (16–44)</td>
</tr>
<tr>
<td>7 (n = 147 M, 144 F)</td>
<td>39</td>
<td>63</td>
<td>162</td>
<td>24</td>
</tr>
<tr>
<td>Total (n = 602 M, 593 F)</td>
<td>55 ± 15</td>
<td>75 ± 6</td>
<td>167 ± 3</td>
<td>27 ± 1</td>
</tr>
</tbody>
</table>

1 All values (except totals) are unweighted \( \bar{x} \) or \( \bar{x} \pm SD \); range in parentheses.

#### TABLE 2

Comparison of body-composition data obtained with the Hologic (Bedford, MA) QDR 4500A and with the criterion method

<table>
<thead>
<tr>
<th>Sample</th>
<th>Criterion method</th>
<th>DXA</th>
<th>DXA – Criterion</th>
<th>DXA</th>
<th>DXA – Criterion</th>
<th>Body mass, DXA – Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n = 22)</td>
<td>TBW (^2)</td>
<td>45.0</td>
<td>2.4 ± 1.4 (^3)</td>
<td>26.6</td>
<td>-1.7 ± 1.5 (^4)</td>
<td>0.7 (^5)</td>
</tr>
<tr>
<td>2 (n = 278)</td>
<td>TBW (^2)</td>
<td>52.2</td>
<td>4.7 ± 1.9 (^4)</td>
<td>24.0</td>
<td>-5.1 ± 2.7 (^4)</td>
<td>-0.2 (^6)</td>
</tr>
<tr>
<td>3 (n = 24)</td>
<td>TBW (^2)</td>
<td>55.4</td>
<td>3.0 ± 2.5 (^4)</td>
<td>23.3</td>
<td>-2.1 ± 2.6 (^4)</td>
<td>0.9 (^6)</td>
</tr>
<tr>
<td>4 (n = 71)</td>
<td>TBW (^2)</td>
<td>50.2</td>
<td>2.0 ± 1.6 (^4)</td>
<td>30.4</td>
<td>-2.4 ± 1.8 (^4)</td>
<td>-0.3 (^6)</td>
</tr>
<tr>
<td>5 (n = 58)</td>
<td>4C (^4)</td>
<td>53.5</td>
<td>3.3 ± 1.8 (^4)</td>
<td>23.5</td>
<td>-2.2 ± 1.8 (^4)</td>
<td>1.1 (^6)</td>
</tr>
<tr>
<td>6 (n = 451)</td>
<td>UWW (^5)</td>
<td>54.2</td>
<td>1.8 ± 3.5 (^4)</td>
<td>23.4</td>
<td>-1.3 ± 3.6 (^4)</td>
<td>0.5 (^6)</td>
</tr>
<tr>
<td>7 (n = 191)</td>
<td>4C (^6)</td>
<td>45.3</td>
<td>1.9 (^7)</td>
<td>17.5</td>
<td>-1.9 (^7)</td>
<td>NA</td>
</tr>
</tbody>
</table>

\(^1\) DXA, dual-energy X-ray absorptiometry; TBW, total body water; 4C, 4-compartment model; UWW, underwater weighing; NA, not available.

\(^2\) Assumes hydration of FFM = 0.73.

\(^3\) Significantly different from 0, \( P < 0.05 \).

\(^4\) Four-compartment model of Withers et al (23).

\(^5\) Densitometry, two-compartment density model of Siri (24).

\(^6\) Four-compartment model of Baumgartner et al (25).

\(^7\) Published analysis showed that DXA underestimated percentage fat.
Protocols

Protocols varied only slightly between laboratories. All participants were measured after an overnight fast and were asked to avoid strenuous exercise on the day before measurement to avoid dehydration. The participants were weighed in a hospital gown or scrubs (data sets 2, 3, 4, and 5) or light clothing, ie, tee shirt and shorts or sweat pants (data sets 1 and 6) and then underwent body-composition analysis with the QDR 4500A and with the criterion method. Further details of the protocols are published elsewhere (12, 21, 26).

Whole-body DXA

A Hologic model QDR 4500A fan-beam X-ray absorptiometer was used to measure body composition at each site. Lean soft tissue mass (LSTM), FM, and bone mineral content (BMC) were assessed by using software versions 8.21, 8.25A, or 8.26A (Whole Body Analysis software), which all use pixel-specific adjustment for beam magnification and the same routine for estimating body composition. Participants were positioned for whole-body scans according to the protocol recommended by the manufacturer. Participants lay supine on the DXA table with limbs close to their bodies. Only those with all body parts in the scan field were included in this study. FFM_{DXA} was the sum of LSTM and BMC.

DXA phantom analysis

To test whether the instruments were comparable, the Hologic whole-body phantom was circulated among laboratories 1 to 5 as well as the 3 NHANES mobile examination centers. At each site, the phantom was scanned 10 times with repositioning of the phantom between each scan; thus, the results included positioning error. The phantom measurements were analyzed and reviewed for accuracy errors by trained staff at the University of California, San Francisco. The mean and SD for the most commonly used variables from each phantom were calculated by using standard methods, including body mass, LSTM, FM, percentage fat, BMD, and BMC. The ratio of LSTM obtained with each instrument to the mean value averaged across all instruments was calculated and compared with the ratio of FFM_{DXA} to FFM_{criterion} for each individual DXA instrument by linear regression.

Criterion methods

Data set 1 was from the Virginia Polytechnic Institute and State University. The criterion method was TBW measured by deuterium dilution. After a baseline venous blood sample was collected, subjects were given a weighed dose of deuterium oxide (99.8 atom%; Isotec Inc, Miamisburg, OH), equivalent to 0.30 g/kg body weight in 100 mL distilled water, followed by a 100-mL distilled water rinse. After a 3-h equilibration period, during which subjects did not ingest anything, a second postdose blood sample was drawn. Plasma samples were purified by diffusion after the method described by Davis et al (27). Plasma samples were purified by incubating equal volumes of plasma and distilled deionized water at 37 °C for 48 h in incubation dishes (Bel-Air Products, Pequannock, NJ). Purified plasma samples were subsequently analyzed for deuterium enrichment by isotope ratio mass spectrometry (MAT 251; Finnigan, Bremen, Germany). TBW was corrected for urinary deuterium losses and reduced by 4% to correct for exchange with protein and carbohydrate during the 3-h equilibration period (28).

Data set 2 was from the energy expenditure subset of the Health, Aging, and Body Composition (Health ABC) Study conducted at the University of Pittsburgh and the University of Tennessee Health Science Center as described elsewhere (29, 30). The criterion method was TBW measured by isotope dilution; =4 g deuterium and 8 g 18O-labeled water were given to fasted subjects after collection of a baseline urine sample. Three additional urine samples were collected in the next 6 h. Enrichments of tracer were measured in the final 2 urine samples relative to baseline by isotope ratio mass spectrometry (Finnigan Delta-S and Delta Plus). Corrections were made for water intake during urine collections, and dilution spaces were reduced by 4% and 0.7% for deuterium and 18O dilution, respectively, and averaged (28).

Data set 3 was from the US Department of Agriculture, Beltsville, MD. The criterion method was TBW measured by deuterium dilution. After an overnight fast, subjects provided a urine sample that was used to measure background isotope enrichments and were then dosed orally with 0.1 g/kg body weight each of deuterium and 18O. The bottle containing the dose was rinsed with 100 mL of deionized water and consumed. After that, the subjects were offered a muffin and juice and were allowed coffee, tea, or water for up to 1 h postdose. Food and beverages were not allowed after the first hour. Saliva samples were collected 4 and 4.5 h postdose. Isotopic enrichment was measured by continuous-flow, isotope ratio mass spectroscopy (Europa Scientific Hydra, Cheshire, United Kingdom). TBW was calculated as the average of the deuterium and 18O dilution spaces calculated from the enrichment of the last saliva sample and corrected for 4% and 0.7% in vivo exchange, respectively (28).

Data set 4 was from the University of Tennessee Health Science Center. The criterion method was TBW measured by deuterium dilution as described elsewhere (29, 30). An oral dose of 4 g deuterium oxide was administered to each participant after a 6–12 h fast. Plasma samples were collected into a dry EDTA-coated tube before and 4 h after the isotope administration. Plasma protein was removed by ultrafiltration, and deuterium enrichment above baseline was measured by isotope ratio mass spectrometry (Finnigan Delta Plus). Subjects were allowed fluid at 1 h after the dose, and corrections were made by subtracting this water intake from TBW, but only in those in whom intake exceeded 0.2 kg. A 1% correction was made for isotope exchange during plasma filtration (30) and 4% for in vivo exchange (28).

Data set 5 was from the University of California, San Francisco, as described elsewhere (31). A four-compartment model was used for the criterion method. TBW was measured by using the same methods as described for data set 4. Body density was measured by underwater weighing (UWW) while the subjects wore a bathing suit. Water temperature was set at 32–35 °C. Five of the most consistent trials (underwater weights within a range of 0.02 kg) from 10 replicates were averaged. Before submersion, residual lung volume was measured in triplicate by using a respirometer (model SVR/PLUS; Collins, Braintree, MA). Total-body mineral mass was calculated from measured total-body BMC in the skeleton by the QDR 4500A. To account for the mineral in nonosseous tissue, total mineral from DXA was multiplied by 1.23. The four-compartment model equation of Lohman (32) was used except as noted above, where total-body mineral was used rather than bone mineral (33).
Data set 6 was from the Life Span Health Research Center at the Wright State University School of Medicine. The criterion method was UWW. Underwater weight was measured in a tank of water (4 ft wide, 6 ft long, and 5 ft deep) at 34 °C. The chair was suspended by 4 load cells whose weights were summed and the weight printed. Residual lung volume was measured on land with a SensorMedics model 2450 (Yorba Linda, CA) Pulmonary Function Laboratory. Weight in air was measured on a Seca scale (Hamburg, Germany) to 0.1 kg. The body density data for each adult participant were converted into FFM by using the Siri equation as described in detail by Guo et al (26).

Data set 7 was extracted from published data (22) and included to show external validity. It was the only other study that met our criteria for comparing the QDR 4500A against TBW, UWW, or a four-compartment model as the criterion method. Briefly, these investigators used the four-compartment model of Baumgartner et al (25). Body density was measured by air plethysmography. TBW was measured by deuterium dilution 3 h after a 10-g oral dose of deuterium oxide using plasma. Deuterium was measured by infrared spectroscopy, and a 5% correction for in vivo isotope exchange was applied. Bone mineral was determined by using the QDR 4500A and multiplied by 1.167 to adjust it to the Lunar DPXL equivalent and again by 1.235 to calculate total-body mineral to match the assumptions used in the development of the four-compartment model (25).

Calculation of FFM

When the criterion method was TBW, FFM was calculated as TBW/0.73. FM was calculated as body mass minus FFM. The selection of 0.73 as the hydration of FFM was based on an extensive review of the literature (Table 3). When the criterion method was the four-compartment model, percentage fat was calculated by using the equations referenced above. FM was calculated as percentage fat/100 multiplied by body mass, and FFM was calculated as the difference.

Statistical analysis

Means and SDs were calculated by using standard unweighted methods. Within-laboratory means for FFM, FM, and body mass from DXA and the criterion method were compared by using a t test. To test for an error in the DXA calibration, regression of FFMDXA on the criterion method results by using the least-squares fit of y on x. Correlations were identified as significant based on the regression coefficient exceeding the critical value for the given df. To test for a constant offset between methods, the intercept was compared with zero and to test for a proportional error, the slope was compared with unity. To determine the correction factor for FFMDXA, FFMDXA was regressed on FFMcriterion while forcing the intercept through zero. In all but 2 of the data sets, the intercept term was not significantly different from zero and thus could be eliminated from the regression. It was eliminated from the other 2 data sets to facilitate comparison between sets. To test for an influence of age, sex, or ethnicity on the DXA\textsubscript{FMM} correction factor, an analysis of variance for these 3 predictors was performed with control for between-laboratory differences by including laboratory as a predictor. Variances were compared by using the F test. Statistical significance required a P value ≤ 0.05. Statistical calculations were performed by using JMP version 4 (SAS Institute Inc, Cary, NC).

RESULTS

The combined data set included 1195 adults who represented a wide range of ages and body sizes (Table 1). The subjects were racially diverse, including 750 whites, 153 African Americans (132 of whom were in sample 2), 1 Asian American, and 291 Asians.

Data were combined for men and women, and the DXA estimate of FFM was compared with that from the criterion method individually for data sets 1–6 (Table 2). FM was 1.8–4.7 kg larger by DXA than by the criterion method, and FM was 1.3–5.1 kg smaller by DXA in laboratories 1–6 (Table 3). DXA-determined body mass was significantly different from scale mass in all laboratories, but the differences included both negative and positive offsets, the largest of which was 1.1 kg. Data on FFM and FM were not directly available from sample 7, but it was reported that the DXA-derived percentage fat was 2 and 4 percentage points smaller in women and men, respectively, than that from the four-compartment criterion method. From this we calculated that FFM averaged 1.9 kg greater by DXA and FM 1.9 kg less by DXA.

FFMDXA was found to have a strong linear relation with FFMcriterion in each of the data sets. The correlation coefficients ranged from 0.972 in data set 4 to 0.991 in data set 5. When FFMDXA was regressed on FFMcriterion, the slopes ranged from 0.909 to 0.967 (Table 4). The average of the individual slopes was 0.946, which indicates that DXA overestimated FFM by 5.4% with an SE of 0.7% compared with the criterion methods. The SE of the slope for data set indicated that the precision of the linear fit was significantly greater for data sets 1 and 3 than for the other data sets (P < 0.05; F test). Of note, however, the variance about the slope within a laboratory (VWL) for any given data set is less than the variance in the slopes (Vtotal) when averaged between data sets (P < 0.01; F test). Calculation of the between–data set (laboratory) variance (\( V_{HL} = V_{total} - \sum V_{WL} \)) indicates that the between–data set SD for the slope was 0.015. This value is more than twice the largest within–data set SE and, therefore, the between–data set (laboratory) variance has a greater influence on the final average across data sets than does the individual variance within a data set.

As a further test of between-laboratory variance, a phantom was exchanged among laboratories for analysis (Table 5). The cross-validation identified small but significant differences between instruments with regard to mass, FM, and BMC. The largest difference between instruments for FFM (LSTM + BMC), however, was only 396 g, or 1.4% of the phantom mass, and thus smaller than the FFM differences between DXA and the criterion methods identified in Table 2. This indicated that the between-laboratory error was probably not due to instrument-to-instrument variation.

We further tested whether the between-laboratory differences observed in the phantom analysis could explain differences in the comparison of FFMDXA and FFMcriterion. We could not compare the absolute errors from the phantom data with those of the human data because of the differences in total mass between the phantom and the humans. Thus, it was necessary to express the phantom data as the ratio of LSTM for each laboratory to the average LSTM for all laboratories so that it could be compared with the percentage error in
the human FFMDXA data for each laboratory. This ratio for the human FFMDXA data for each laboratory.

<table>
<thead>
<tr>
<th>Criterion method</th>
<th>Post hoc correction</th>
<th>Race</th>
<th>Sex</th>
<th>Age</th>
<th>Miscellaneous</th>
<th>FFM hydration</th>
<th>Reference</th>
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<tbody>
<tr>
<td>IVNA —</td>
<td>NA</td>
<td>M</td>
<td>22–92</td>
<td>Malnourished</td>
<td>73.2 ± 2.4³,⁴</td>
<td>Beddoo et al (34)</td>
<td></td>
</tr>
<tr>
<td>IVNA —</td>
<td>NA</td>
<td>F</td>
<td>20–88</td>
<td>Malnourished</td>
<td>75.0 ± 3.1⁴</td>
<td>Beddoo et al (34)</td>
<td></td>
</tr>
<tr>
<td>IVNA —</td>
<td>NA</td>
<td>M</td>
<td>20–58</td>
<td></td>
<td>71.1 ± 1.2</td>
<td>Beddoo et al (34)</td>
<td></td>
</tr>
<tr>
<td>IVNA —</td>
<td>NA</td>
<td>F</td>
<td>19–59</td>
<td></td>
<td>72.6 ± 1.5</td>
<td>Beddoo et al (34)</td>
<td></td>
</tr>
<tr>
<td>4C H exchange</td>
<td>W</td>
<td>M</td>
<td>65–94</td>
<td></td>
<td>74.5 ± 4.5</td>
<td>Baumgartner et al (25)</td>
<td></td>
</tr>
<tr>
<td>4C H exchange</td>
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<td>F</td>
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<td>74.6 ± 3.9</td>
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<td></td>
</tr>
<tr>
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<td>M</td>
<td>18–59</td>
<td></td>
<td>73.3 ± 2.2</td>
<td>Fuller et al (35)</td>
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</tr>
<tr>
<td>4C H exchange</td>
<td>NA</td>
<td>F</td>
<td>18–59</td>
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<td>74.5 ± 1.9</td>
<td>Fuller et al (35)</td>
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<tr>
<td>3C H exchange</td>
<td>W</td>
<td>M</td>
<td>22–39</td>
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<td>70.2 ± 1.0</td>
<td>Hewitt et al (36)</td>
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<tr>
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<td>W</td>
<td>F</td>
<td>22–39</td>
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<td>69.9 ± 1.3</td>
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<td>Hewitt et al (36)</td>
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<td>NA</td>
<td>M</td>
<td>23–72</td>
<td></td>
<td>72.5 ± 0.8</td>
<td>Ryde et al (37)</td>
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</tr>
<tr>
<td>IVNA —</td>
<td>NA</td>
<td>F</td>
<td>23–72</td>
<td></td>
<td>72.2 ± 0.8</td>
<td>Ryde et al (37)</td>
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<tr>
<td>4C H exchange</td>
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<td>M</td>
<td>30 ± 4</td>
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<td>M</td>
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<td>Mazariegos et al (38)</td>
<td></td>
</tr>
<tr>
<td>4C H exchange</td>
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<td>M</td>
<td>28 ± 4</td>
<td>Runners</td>
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<td>Penn et al (39)</td>
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<td>M</td>
<td>28 ± 4</td>
<td></td>
<td>72.9 ± 1.6</td>
<td>Penn et al (39)</td>
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</tr>
<tr>
<td>3C (Siri) —</td>
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<td>F</td>
<td></td>
<td>Gravid (3rd trimester)</td>
<td>76.2</td>
<td>Calalano et al (40)</td>
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<tr>
<td>4C H exchange</td>
<td>W</td>
<td>M</td>
<td>24 ± 4</td>
<td>Resistance training</td>
<td>74.4 ± 1.2³</td>
<td>Modlesky et al (41)</td>
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<tr>
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<td>W</td>
<td>M</td>
<td>24 ± 4</td>
<td></td>
<td>71.2 ± 2.0</td>
<td>Modlesky et al (41)</td>
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<tr>
<td>4C H exchange</td>
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<td>M</td>
<td>19–27</td>
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<td>72.5 ± 1.0</td>
<td>Bergsma-Kadijk et al (42)</td>
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<td>M</td>
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<td>Asian</td>
<td>M</td>
<td></td>
<td></td>
<td>70.4 ± 2.4</td>
<td>Borgounia et al (43)</td>
<td></td>
</tr>
<tr>
<td>3C (Siri) —</td>
<td>Asian</td>
<td>F</td>
<td></td>
<td></td>
<td>71.9 ± 2.4</td>
<td>Borgounia et al (43)</td>
<td></td>
</tr>
<tr>
<td>4C H exchange</td>
<td>F</td>
<td>30 ± 4</td>
<td>&gt;3 mo postpartum</td>
<td></td>
<td>73.3 ± 2.0</td>
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<tr>
<td>4C H exchange</td>
<td>AA</td>
<td>M</td>
<td>20–94</td>
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<td>74.1 ± 3.2</td>
<td>Visser et al (45)</td>
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</tr>
<tr>
<td>4C H exchange</td>
<td>W</td>
<td>M</td>
<td>20–94</td>
<td></td>
<td>74.6 ± 2.8</td>
<td>Visser et al (45)</td>
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</tr>
<tr>
<td>4C H exchange</td>
<td>AA</td>
<td>F</td>
<td>20–94</td>
<td></td>
<td>75.3 ± 3.6</td>
<td>Visser et al (45)</td>
<td></td>
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<tr>
<td>4C —</td>
<td>NA</td>
<td>M</td>
<td>69 ± 7</td>
<td></td>
<td>74.7 ± 3.8</td>
<td>Goran et al (9)</td>
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</tr>
<tr>
<td>4C —</td>
<td>NA</td>
<td>F</td>
<td>69 ± 7</td>
<td></td>
<td>72.4 ± 4.6</td>
<td>Goran et al (9)</td>
<td></td>
</tr>
<tr>
<td>4C —</td>
<td>NA</td>
<td>M</td>
<td>26 ± 6</td>
<td>Athletically trained</td>
<td>71.1</td>
<td>Withers et al (23)</td>
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<td>F</td>
<td>26 ± 6</td>
<td>Athletically trained</td>
<td>70.8</td>
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<tr>
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<td>M</td>
<td>26 ± 6</td>
<td></td>
<td>70.5</td>
<td>Withers et al (23)</td>
<td></td>
</tr>
<tr>
<td>4C —</td>
<td>NA</td>
<td>F</td>
<td>26 ± 6</td>
<td></td>
<td>71.4</td>
<td>Withers et al (23)</td>
<td></td>
</tr>
<tr>
<td>4C —</td>
<td>NA</td>
<td>M</td>
<td>&gt;60</td>
<td>Young adult</td>
<td>73.2 ± 2.4</td>
<td>Ritz et al (46)</td>
<td></td>
</tr>
<tr>
<td>4C —</td>
<td>NA</td>
<td>M</td>
<td>&gt;60</td>
<td>Young adult</td>
<td>73.4 ± 2.4</td>
<td>Ritz et al (46)</td>
<td></td>
</tr>
<tr>
<td>4C —</td>
<td>Asian</td>
<td>M</td>
<td>23 ± 4</td>
<td></td>
<td>73.2 ± 1.7</td>
<td>Werkman et al (47)</td>
<td></td>
</tr>
<tr>
<td>4C —</td>
<td>Asian</td>
<td>F</td>
<td>23 ± 4</td>
<td></td>
<td>72.8 ± 1.5</td>
<td>Werkman et al (47)</td>
<td></td>
</tr>
<tr>
<td>4C —</td>
<td>W</td>
<td>M</td>
<td>23 ± 4</td>
<td></td>
<td>72.9 ± 1.9</td>
<td>Werkman et al (47)</td>
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<tr>
<td>4C —</td>
<td>W</td>
<td>F</td>
<td>23 ± 4</td>
<td></td>
<td>74.2 ± 1.4</td>
<td>Werkman et al (47)</td>
<td></td>
</tr>
</tbody>
</table>

¹ IVNA, in vivo neutron activation; 4C, four-compartment model; 3C, three-compartment model; AA, African American; W, white; NA, not available.

² Recalculated from published data to include a correction for hydrogen tracer exchange with nonaqueous material of 4.2% (48).

³ ± SD (all such values).

⁴ Significantly different from the study’s identified control group, *P < 0.05.

⁵ Adjusted for between-individual differences in bone mineral density.

The subjects were not equally stratified for sex, race, and age across the laboratories. To test whether the slope for the FFMDXA method for those data sets in which comparisons between several criterion methods could be made (Table 6). This was only possible in 2 data sets, however. The slopes and SDs (0.952 ± 0.019) for the various criterion methods from these 2 data sets were not different from those for the between-laboratory variance (0.945 ± 0.016; Table 4).

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A correction factor for QDR 4500A estimates of FFM and FM was determined by using the assumption that BMC obtained from the QDR 4500A is correct and should not be adjusted. Corrected FFM_{DXA} was obtained by multiplying LSTM by the determined correction factor. Corrected FM was then calculated by subtracting the sum of LSTM and BMC from total body weight determined by DXA as follows:

\[
\text{LSTM}_{\text{corrDXA}} = 0.946 \times \text{LSTM}_{\text{DXA}}
\]

\[
\text{FM}_{\text{corrDXA}} = \text{DXA weight} - (0.946 \times \text{LSTM}_{\text{DXA}} + \text{BMC})
\]

Percentage fat = 100(\text{FM}_{\text{corrDXA}}/\text{weight}_{\text{DXA}})

BMC was not corrected.

### TABLE 4
Summary of the proportional error in fat-free mass measured by dual-energy X-ray absorptiometry compared with the proportional error in that measured with criterion method based on linear regression analysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Criterion</th>
<th>Slope</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ((n = 22))</td>
<td>TBW</td>
<td>0.946(^2)</td>
<td>0.006</td>
</tr>
<tr>
<td>2 ((n = 278))</td>
<td>TBW</td>
<td>0.909(^2)</td>
<td>0.002</td>
</tr>
<tr>
<td>3 ((n = 24))</td>
<td>TBW</td>
<td>0.941(^3)</td>
<td>0.007</td>
</tr>
<tr>
<td>4 ((n = 71))</td>
<td>TBW</td>
<td>0.957(^3)</td>
<td>0.003</td>
</tr>
<tr>
<td>5 ((n = 58))</td>
<td>4C</td>
<td>0.950(^2)</td>
<td>0.003</td>
</tr>
<tr>
<td>6 ((n = 451))</td>
<td>UWW</td>
<td>0.967(^2)</td>
<td>0.003</td>
</tr>
<tr>
<td>7 ((n = 291))</td>
<td>4C</td>
<td>0.946(^4)</td>
<td>0.002</td>
</tr>
<tr>
<td>Total</td>
<td>—</td>
<td>0.946(^4)</td>
<td>0.007(^4)</td>
</tr>
</tbody>
</table>

\(^{1}\) TBW, total body water; \(^{2}\) Four-compartment model; \(^{3}\) Intercept was significantly different from 0 before forcing the regression through 0. \(^{4}\) Average and SE of the mean for the slopes for the 7 data sets.

### TABLE 5
Interlaboratory comparison of the Hologic (Bedford, MA) whole-body dual-energy X-ray absorptiometry (DXA) phantom.

<table>
<thead>
<tr>
<th>Sample and model</th>
<th>FM</th>
<th>FM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXA (^2)</td>
<td>53.5 \pm 12.2</td>
<td>23.5 \pm 7.4</td>
</tr>
<tr>
<td>TBW (^3)</td>
<td>50.1 \pm 10.7 (0.935)</td>
<td>25.9 \pm 8.0</td>
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<tr>
<td>UWW (^4)</td>
<td>50.4 \pm 12.5 (0.947)</td>
<td>25.4 \pm 8.4</td>
</tr>
<tr>
<td>4C (^5)</td>
<td>50.3 \pm 11.5 (0.932)</td>
<td>25.5 \pm 8.1</td>
</tr>
<tr>
<td>4C (^6)</td>
<td>50.9 \pm 11.8 (0.953)</td>
<td>25.0 \pm 8.0</td>
</tr>
<tr>
<td>(^7) ((n = 291))</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{1}\) All values are \(\bar{x} \pm SD\); \(^{2}\) Slope of the linear relation \(\text{FFM}_{\text{criterion}} = \text{FFM}_{\text{DXA}}\) in parentheses. TBW, total body water; UWW, under-water weighing; \(^{3}\) Measured with the Hologic (Bedford, MA) QDR 4500A. \(^{4}\) FM = TBW/0.73. \(^{5}\) Siri equation (24). \(^{6}\) Model of Withers et al (49). \(^{7}\) Calculated from published data of Deurenberg-Yap et al (22).
We compared body-composition estimates obtained from the 7 Hologic QDR 4500A DXA instruments with those determined with several criterion methods in 7 independent data sets. In each, we found that \( \text{FFM}_{\text{DXA}} \) was larger than that of the criterion method. Because of the consistency of the results across criterion methods, we concluded that \( \text{FM} \), as measured with the QDR 4500A DXA, was significantly overestimated. Regression analysis indicated that the difference from the value determined with the criterion methods was a proportional difference; therefore, a proportional correction factor is recommended.

One potential criticism of our findings was that 4 of the 7 data sets used TBW as the criterion method. The use of TBW requires an assumption of a constant hydration of \( \text{FM} \). Wang et al (49) recently provided a theoretical basis for variation in the hydration of \( \text{FM} \) in healthy adults and, in so doing, predicted a maximal range for the individual hydration factors of 0.66 to 0.77. Our review of the literature indicated that group mean values are less variable than those predicted by Wang et al (49), ranging from 0.70 to 0.75 in healthy adults (Table 1). Our assumed value of 0.73 is at the center of these ranges. Individual studies within this compilation indicate that hydration of \( \text{FM} \) is a few percentage points larger during pregnancy, in severe protein malnutrition, and in muscle builders (34, 40, 41); these observations are consistent with the theoretical basis of hydration reviewed by Wang et al (49). After exclusion of these 3 subsets, we performed a meta-analysis to determine whether there were effects of age in adulthood, sex, or ethnicity on hydration of \( \text{FM} \). The average hydration of \( \text{FM} \), excluding the 3 groups listed above with significant increases in hydration, was 72.6 ± 1.4% (between-study SD) with no significant effect of age in adulthood, sex, or ethnicity. These lines of evidence support our use of a hydration factor of 0.73 for our populations.

We also questioned the use of different criterion methods in our analysis. To address this concern, we performed further data analyses in 2 of our data sets that had measurements that permitted the calculation of \( \text{FFM} \) by several different criterion methods (Table 6). The mean \( \text{FFM} \) was similar among the various criterion methods, and the slopes between DXA and the criterion method were all <1.0. Moreover, the within-criterion method variances for the slopes were comparable with those for TBW. This provides further support for the use of multiple criterion methods and substantiates the use of TBW as a criterion method for assessing the accuracy of the calibration of the QDR 4500A.

Our use of an unweighted mean to calculate the correction factor for \( \text{FM} \) rather than a weighted mean for sample size could also be questioned. Our choice of the unweighted mean was based on the between-laboratory variance being larger than the within-laboratory variance. Both the literature review of the \( \text{FFM} \) hydration data and the data in Tables 4 and 6 indicate a larger between-laboratory variation than would be predicted from the SE for the within-laboratory mean. This indicates that there was a systematic difference in the criterion methods between laboratories and that this difference became limiting for the interlaboratory comparison. Thus, we used the between-laboratory average for the slope of \( \text{FFM}_{\text{DXA}} \) on \( \text{FM}_{\text{criterion}} \) without weighting, despite greatly different numbers of participants in the data sets.

It is also possible that the between-laboratory variance may have resulted from between-instrument variance of the DXA instruments. A common whole-body phantom was circulated among the laboratories to determine interlaboratory differences for the assessment of \( \text{FFM} \). The results of phantom data analysis showed no difference between the DXA instruments used in this calibration study. Because the circulation of the phantom was done 1–5 y after the human data were collected, it may not represent the actual accuracy of the particular instrument during the collection of the human data. We speculated, however, that the differences that existed between these instruments during the period of data collection were not any greater than those measured during the phantom analysis for the following reasons. Although calibration of a DXA instrument can be altered as a result of a major repair to the DXA hardware or the upgrade of software, none of the laboratories reported any major repair to their DXA instrument nor were there any differences in the software versions used to collect the human or phantom data. It should also be noted that the 3 NHANES DXA instruments being used to collect the national data were found to provide estimates of \( \text{FFM} \) similar to those obtained by the laboratories used to determine our correction factor (Table 5). This finding supports the use of the above-suggested corrections for the NHANES data.

The error for the \( \text{FFM}_{\text{DXA}} \) value for the QDR 4500A may appear surprising in light of the large number of publications that have found DXA to be an accurate means of assessing \( \text{FFM} \) and \( \text{FM} \) in adults. Many of these validations, however, were performed with the use of pencil-beam instruments. The QDR 4500A is a fan-beam instrument and thus involves a correction for beam magnification and a new software routine for converting the X-ray absorption data to body composition. Beam magnification has been shown to influence the measurement of \( \text{FFM} \) and \( \text{FM} \) by DXA (13). This and the use of the new software appear to have introduced a modest systematic error in the \( \text{FFM} \) calibration that requires correction. This error is specific to the QDR 4500A and these software versions. Other fan-beam instruments require independent validation to determine whether they too are subject to systematic bias.

The calibration of the QDR 4500A is of particular importance because it is currently being used to acquire body-composition data for a nationally representative sample of individuals in the United States as part of the NHANES. Because these data may be used to make national policy decisions, comparisons over time within the United States, comparisons between countries, and comparisons of study cohorts with the NHANES sample, an assessment of the accuracy of the QDR 4500A was critical. Based on our findings of a biologically significant bias in the QDR 4500A, this calibration was implemented in NHANES 1999–2004 data before its release. We suggest that others who use the QDR 4500A should also use this correction for \( \text{FFM} \), \( \text{FM} \), and percentage fat.

Technical information regarding the QDR 4500A absorptiometer was provided by Thomas L Kelly of Hologic Inc.

LGB conceived and organized the combined effort of the authors. DAS, FAT, and TGL organized the data and data analysis and prepared the first draft of the manuscript. DBJ, WCC, CPE, TE, TBH, SBH, HCL, HM, JS, and RMS provided the data sets and edited the manuscript. None of the authors reported having a conflict of interest.

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Evening intake of α-lactalbumin increases plasma tryptophan availability and improves morning alertness and brain measures of attention

C Rob Markus, Lisa M Jonkman, Jan HCM Lammers, Nicolaas EP Deutz, Marielle H Messer, and Nienke Rigtoring

ABSTRACT

Background: Brain serotonin function is thought to promote sleep regulation and cognitive processes, whereas sleep abnormalities and subsequent behavioral decline are often attributed to deficient brain serotonin activity. Brain uptake of the serotonin precursor tryptophan is dependent on nutrients that influence the availability of tryptophan via a change in the ratio of plasma tryptophan to the sum of the other large neutral amino acids (Trp:LNAA).

Objective: We tested whether evening consumption of α-lactalbumin protein with an enriched tryptophan content of 4.8 g/100 g increases plasma Trp:LNAA and improves alertness and performance on the morning after sleep, particularly in subjects with sleep complaints.

Design: Healthy subjects with (n = 14) or without (n = 14) mild sleep complaints participated in a double-blind, placebo-controlled study. The subjects slept at the laboratory for 2 separate nights so that morning performance could be evaluated after an evening diet containing either tryptophan-rich α-lactalbumin or tryptophan-low placebo protein. Evening dietary changes in plasma Trp:LNAA were measured. Behavioral (reaction time and errors) and brain measures of attention were recorded during a continuous performance task.

Results: Evening α-lactalbumin intake caused a 130% increase in Trp:LNAA before bedtime (P = 0.0001) and modestly but significantly reduced sleepiness (P = 0.013) and improved brain-sustained attention processes (P = 0.002) the following morning. Only in poor sleepers was this accompanied by improved behavioral performance (P = 0.05).

Conclusion: Evening dietary increases in plasma tryptophan availability for uptake into the brain enhance sustained alertness early in the morning after an overnight sleep, most likely because of improved sleep.

KEY WORDS Sleep complaints, continuous performance task, attention, α-lactalbumin, tryptophan, event-related brain activity

INTRODUCTION

Reduced alertness after poor sleep often deteriorates cognitive-behavioral functioning (1). Most of the observed negative effects are on performance that requires sustained attention, which are recognized by vigilance tasks that are repetitive, simple, and of long duration (2, 3). Vigilance studies indicate that sleepiness progressively reduces attention and efficient stimulus detection (4), as evidenced by lower behavioral responsiveness and by reduced parietal event–related brain activity that occurs ≈300 ms after stimulus onset (P300), which indicates reduced attention processing (5). Consequently, reduced vigilance after poor sleep often leads to dramatic catastrophes and traffic accidents (6). Moreover, sleep disturbances often precede affective complaints and depressive symptoms (7). From a prevention perspective, it is important to search for mechanisms that mediate the effect of poor sleep on cognitive performance.

The effects of poor sleep on performance may be partly mediated by a biochemical imbalance of brain serotonin (5-hydroxytryptamine, or 5-HT). Brain 5-HT seems to be involved in the regulation of sleep and cognitive processes (8, 9), and sleep abnormalities and cognitive decline in clinical populations are partly attributable to deficient brain 5-HT activity (10, 11). Accordingly, reduced 5-HT concentrations resulting from the exhaustion of its plasma precursor tryptophan was found to provoke sleep abnormalities seen in depression (10, 12), whereas increases in available plasma tryptophan for uptake into the brain improved sleep in different subjects (9, 13, 14). These sleep-promoting effects of tryptophan may be stronger in subjects with sleep complaints, because they are vulnerable to the sleep-reducing effects of tryptophan depletion (12). In further support, sleep complaints are often accompanied by stress and emotional decline (15, 16) that, in turn, may alter 5-HT receptor sensitization (17, 18) and subsequently increase tryptophan and 5-HT vulnerability (18). Even though these findings suggest positive relationships between tryptophan availability and sleep, beneficial effects of increases in evening tryptophan on morning alertness have not yet been investigated. Instead, studies exclusively focused on the effects of tryptophan alterations on electroencephalographic (EEG) sleep recordings without exploring its relevance to morning performance.

Available brain tryptophan and 5-HT in humans is thought to rise with the intake of L-tryptophan or carbohydrates, which raise

1 From the Departments of Experimental Psychology (CRM, MHM, and NR) and Neurocognition (LMJ) and the Biomedical Center (NEPD), University of Maastricht, Maastricht, Netherlands, and TNO Quality of Life, Zeist, Netherlands (JHCML).

2 The α-lactalbumin protein was provided by Davisco Foods International (Eden Prairie, Minnesota).

3 Reprints not available. Address correspondence to CR Markus, University of Maastricht, Department of Experimental Psychology, PO Box 6200 MD Maastricht, Netherlands. E-mail: r.markus@psychology.unimaas.nl. Received July 1, 2004. Accepted for publication January 14, 2005.

the ratio of plasma tryptophan to large neutral amino acids (Trp:LNAA) (19, 20). These methods, however, may have physical side effects, and carbohydrate ingestion showed no or only small behavioral effects due to modest tryptophan alterations (21, 22). An alternative method for raising tryptophan availability, and probably 5-HT, may be the intake of α-lactalbumin (A-LAC) protein, which contains the highest tryptophan content of all food-protein sources (23). Recently, it was shown that A-LAC containing 1.3 g/100 g tryptophan causes a 48% increase in plasma Trp:LNAA (18). In addition, A-LAC was found to increase brain 5-HT concentrations (24) and to reduce sleep disturbances in rats (25). The present study investigated whether the evening intake of A-LAC may improve morning performance in subjects with sleep complaints. To enhance the effects of A-LAC on Trp:LNAA, A-LAC protein was used with a greater tryptophan content (4.8 g/100 g) than that used in previous studies (18).

SUBJECTS AND METHODS

Subjects
Dutch University students (n = 235) filled out the Dutch Groningen Sleep Questionnaire (GSQ; 26) and a questionnaire package concerning personal details. From the highest quartile of the GSQ score (3 ± SD: 22 ± 2; range: 20–27), 14 subjects (7 men and 7 women aged 22 ± 2 y) were selected for the poor-sleepers group; from the lowest quartile of the GSQ score (16 ± 1; range: 15–17), 14 subjects (7 men and 7 women aged 22 ± 3 y) were selected for the “good sleepers” group. Exclusion criteria for participation were chronic and current illness; a history of psychiatric or medical illness; medication use; metabolic, hormonal, or intestinal diseases; irregular diets or deviant eating habits; excessive use of alcohol, cigarettes, coffee, or drugs; allergy to milk products; and pregnancy as assessed by health and lifestyle questionnaires. All subjects who participated in the experiment had a body mass index (BMI; in kg/m²) in the normal range (BMI: 19–26), were nonsmokers, and were not allowed to drink alcohol or take any drug for 2 d before and during the experiment. The study was approved by the Medical Ethics Committee (MEC 03-0625) of the Academic Hospital Maastricht (Maastricht, Netherlands) and complied with the requirements of the European Council of Good Clinical Practice adopted by the 52nd World Medical Association General Assembly, Edinburgh, United Kingdom (October 2000). All subjects gave their informed consent to participate in the experiment.

Methods
The experimental procedure was conducted according to a double-blind, placebo-controlled design, and the data analysis was conducted without knowledge of the subject’s assignment and dietary condition.

During 2 experimental sessions, subjects with and without sleep complaints stayed at the laboratory for an overnight sleep for the monitoring of their early morning alertness and mental functioning under task-related EEG-ERP registration, after either an evening diet containing tryptophan-enriched A-LAC or standard protein (placebo). The diets were isonenergetic and contained equal amounts of protein, carbohydrate, and fat. The order of presentation of the A-LAC and placebo diets was counterbalanced between subjects. Both experimental sessions were separated by a 3- or 4-wk period to allow for the control of the women’s menstrual cycles, whereas women using oral contraceptives participated during the time when they actually consumed the contraception pill. Two weeks before the start of the experiment, all subjects were invited to the laboratory to familiarize themselves with the sleep conditions and experimental procedures.

For each experimental session, 2 subjects arrived at the laboratory at 1800 (see Figure 1 for an overview). The subjects were instructed to consume a standardized lunch at 1230, for which they received precise descriptions and a food diary, and to fast from 1300 onward (only water or tea without sugar was permitted). After their arrival, the subjects consumed an evening meal together with an A-LAC or placebo milk shake at 1830, which was followed by a second milk shake 1 h later (at 1930). Two hours after intake of the second drink, a blood sample was taken and collected in a tube. Then, subjects were allowed to watch television or to read in a private guest room and also conducted a computer test as part of another study. At 2250, the subjects filled in a computerized Stanford Sleepiness Scale (27) and then went to bed at 2300 (lights off) in a private bedroom until 0700 in the morning (lights on). After showering, the subjects filled in a second sleepiness scale and were brought into the laboratory. After the EEG electrodes were connected, the subjects were seated in front of a computer screen in an electrically shielded and soundproof cabin and conducted a continuous performance task (CPT). Before the CPT started, the subjects first had to run through a practice session until a predetermined criterion was reached (>80% hits and <500 ms). After completion of the CPT, the electrodes were disconnected and the subjects were allowed to go home.

Diets
On both experimental evenings, an isonenergetic diet (Knorr Orienty Nasi; Unilever Bestfoods, Rotterdam, Netherlands) providing 325 kcal (13% of energy as protein, 86% of energy as carbohydrate, and 1% of energy as fat) was provided to the subjects. The 2 dietary conditions were similar, with the exception of an additional milk shake in which the protein sources differed. The milk shake for the experimental diet contained 20 g tryptophan-enriched (4.8 g/100 g tryptophan) A-LAC protein (Davisco Foods International, Eden Prairie, MN) and the milk shake of the placebo diet contained 20 g (1.4 g/100 g tryptophan) sodium caseinate (DMV International, Veggel, Netherlands). The milk shakes were prepared by mixing the A-LAC or placebo protein powder with 7 g strawberry milk shake mix (Nesquik; Nestlé, Vevey, Switzerland) and 200 mL water. The first milk shake was served with the meal (1830), and the second milk shake was served 1 h later (at 1930). A research assistant blind to the dietary conditions supervised the dietary intake to make sure that all foods were consumed within 20 min. The amino acid profile of the protein sources and the nutrient composition of the milk shakes are given in Table 1.

Measurements
Sleepiness
Changes in sleepiness were measured by using the Stanford Sleepiness Scale (27), which was offered on a computer screen and has a 7-point interval scale ranging from “strongly disagree” to “strongly agree.” The first scale was offered 10 min before the subjects went to bed (at 2250), and a second scale was offered the
The following morning 10 min after the subjects awoke (at 0710). The sleepiness scale comprises 7 statements concerning feelings of alertness ranging from “feeling active and vital” (score of 1) to “almost dreaming, sleep onset will be soon” (score of 7). The Stanford Sleepiness Scale is particularly sensitive to changes in alertness that are caused by poor sleep and sleep loss (28).

Continuous performance task

The CPT was used to measure changes in vigilance (29). A series of randomly presented letters (A, E, H, K, L, and X) was presented at the center of the computer screen. The subjects were instructed to push a button as quickly as possible when the letter X appeared on the screen, but only when it was preceded by the letter A (A-X sequence, target condition). When an A was followed by another letter (A-not-X, nontarget condition), the subjects were instructed to not respond. The CPT was administered in 3 time blocks of ≈4-min duration, consisting of 264 trials each, to measure sustained attention. Each block contained 24 A-X sequences and 24 A-other sequences, and 24 X, K, E, H, and L letters were presented randomly alone without a preceding A. Each trial lasted 950 ms, including stimulus duration of 150 ms and a fixed interstimulus interval of 800 ms, and the total task duration was ≈13 min (3 × 4.18 min). Before the task onset, the subjects had to practice to reach sufficient performance (minimal 80% hits and a reaction time (RT) of ≈500 ms), which was generally accomplished within 3–4 min. Stimulus presentation and acquisition of behavioral data were controlled by Experimental Run Time System software (ERTS, version 3.18; Berisoft Corp, Frankfurt, Germany). For each subject, the number of hits (correct A-X responses), misses (missed A-X responses), and false alarms (A-other) as well as the mean RTs during the hits were computed as behavioral measures of (sustained) attention efficiency.

Electroencephalographic recordings

During the CPT, EEG activity was registered continuously from tin electrodes by means of an electro cap from the midline scalp positions, of which only the signals at the parietal electrode (Pz) were analyzed for the purpose of this study. Both horizontal and vertical electro-oculographic (EOG) activity was recorded with the use of 2 tin electrodes attached to the outer canthi of both eyes (horizontal EOG) and from 2 infraorbital and supraorbital tin electrodes placed in line with the pupil of the left eye (vertical EOG). A ground electrode was attached to the middle of the
showed a significant effect of diet (subjects factor and “group” as the between-subjects factor). Plasma Trp:LNAA showed a significant effect of diet (subjects factor and “group” as the between-subjects factor).

Offline, the continuous EEG was divided into epochs from 200 ms prestimulus to 952 ms poststimulus, filtered with a 30-Hz (24 dB/Oct) digital low-pass filter and baseline corrected with a 200-ms prestimulus interval. EEG signals were corrected for vertical EOG artifacts by means of an eye-movement correction algorithm (30), and all EEG epochs containing artifacts exceeding ± 75 μV were removed. Averaged parietal ERPs were computed for individual subjects in each of the 3 CPT blocks, only for target trials to which subjects responded correctly. In the ERP, peak amplitudes and latencies of the P300 component were scored relative to baseline at Pz in a 300–550 ms window.

Biochemical analyses

Blood samples were collected in 5-mL evacuated tubes containing sodium heparin and centrifuged at 2850 × g (5000 rpm) for 5 min at 4 °C. Subsequently, the supernatant fluid (100 μL) was mixed with 4 mg sulfasalicylic acid and stored at −80 °C until analyzed. Amino acid analysis in plasma was conducted with HPLC with the use of a 2–3-μm Bischof Spherisorb ODS II column (31). Plasma Trp:LNAA was calculated by dividing the plasma tryptophan concentration by the sum of the other large neutral amino acids, ie, valine, isoleucine, leucine, tyrosine, and phenylalanine.

Experimental design and statistical analysis

The main research question formulated in the introduction was analyzed by means of univariate repeated-measures analysis of variance (ANOVA) by using the general linear model (SPSS 7.5 for WINDOWS; SPSS Inc, Chicago) with one between-subjects factor “group” (poor sleepers compared with good sleepers, as independent variables) and one within-subjects factors “diet” (A-LAC compared with placebo, as independent variables) on changes in plasma Trp:LNAA and sleepiness and behavioral (RT and errors) and ERP changes during the CPT as dependent variables. For the effect on behavioral and ERP changes during the CPT across the 3 time blocks, multivariate ANOVA also included “block” as a within-subjects factor with first- and second-order polynomial contrasts. Although we counterbalanced for the sequence of dietary condition (order of diet), this factor was incorporated as a covariant when a significant main or interaction effect was found. This was the case for the ERP P300 amplitude and latency. Significant results shown with these procedures were further examined by post hoc tests. Huynh-Feldt– or Greenhouse-Geisser–corrected P values, their corresponding epsilons, and the original (ie, uncorrected) df are reported when the sphericity assumption was not met. All statistics were evaluated at a significance level of 5%. Data are reported as means ± SDs.

RESULTS

Plasma Trp:LNAA

Repeated-measures ANOVA with “diet” as the within-subjects factor and “group” as the between-subjects factor showed a significant effect of diet (P < 0.0001), which indicated a significant (130%) increase in plasma Trp:LNAA after A-LAC as compared with placebo. As shown in Figure 2, the mean plasma Trp:LNAA was higher after the A-LAC diet condition (0.25 ± 0.02) than after the placebo diet condition (0.11 ± 0.03). There was no significant effect of group or an interaction between diet and group.

Sleepiness

Repeated-measures ANOVA with diet as the within-subjects factor and group as the between-subjects factors on evening sleepiness showed no significant effect (P > 0.2). A second analysis with diet as the within-subjects factor and group as the between-subjects factor on morning sleepiness showed a significant effect of diet (P = 0.013). As shown in Figure 3, all subjects were less sleepy in the morning after evening intake of A-LAC (2 ± 0.1) than after placebo intake (3 ± 0.1). There was no significant effect of group or an interaction between diet and group.

Continuous performance task

Behavioral findings

Repeated-measures ANOVA with diet and block as within-subjects factors and group as the between-subjects factor on RT showed a significant effect of block (P = 0.045) and a significant interaction effect of diet × block × group (P = 0.05), which originated from a significant quadratic polynomial contrast (P = 0.014). As indicated in Figure 4, RT was lower in the poor-sleepers group during the second block (from 369 ± 51 to 379 ± 53 ms) and higher at the end of the task during the third block (401 ± 40 ms; P = 0.014) after the A-LAC diet, whereas there were no significant differences after the placebo diet (P > 0.5). This effect or any other effect was not found in the good-sleepers group.

To see whether this interaction effect on RT was accompanied by alterations in the number of errors, we also conducted an
The end of the task (block 3) after the evening intake of a tryptophan-rich α-lactalbumin (A-LAC) diet or a casein (placebo) diet in healthy subjects with (n = 14; ■) or without (n = 14; □) mild sleep complaints. Repeated-measures ANOVA showed a significant main effect of diet (P = 0.013). *Significantly different from placebo, P < 0.05. The mean difference between diets was significant (P = 0.013) when both groups were pooled. There were no significant differences between the subjects with or without sleep complaints.

ANOVA on the number of missed A-X responses. The analysis showed no significant interaction effect of diet × block × group (P > 0.2). However, as indicated in Figure 5, the analysis on the number of false alarms (A-other) did show a significant interaction of diet × block × group (P = 0.008), which indicated a significant linear reduction in the number of false alarms across blocks after the A-LAC diet in poor sleepers (P = 0.048) but not in good sleepers (P > 0.7).

FIGURE 3. Mean (±SEM) changes in sleepiness scores after an overnight sleep following the evening intake of a tryptophan-rich α-lactalbumin (A-LAC) diet or a casein (placebo) diet in healthy subjects with (n = 14; ■) or without (n = 14; □) mild sleep complaints. Repeated-measures ANOVA showed a significant main effect of diet (P = 0.013). *Significantly different from placebo, P < 0.05. The quadratic trends were significantly different between treatments (P = 0.002) but not in the good sleepers.

ERP findings

Repeated-measures ANOVA with diet and block as within-subjects factors and group as the between-subjects factor on parietal P300 amplitudes showed a significant effect of diet (P < 0.002), which indicated that morning P300 amplitude was significantly higher after the A-LAC diet (23 ± 6 μV) than after the placebo diet (19 ± 6 μV) (Figure 6). No other main or interaction effects were found.

Repeated-measures ANOVA on parietal P300 latency showed a significant effect of diet (P = 0.002) and a significant interaction of diet × block × group (P < 0.04). Further post hoc testing

FIGURE 5. Mean (±SEM) changes in the number of missed A-X responses during a continuous performance task after an overnight sleep following the evening intake of a tryptophan-rich α-lactalbumin diet (■) or a casein (placebo) diet (□) in healthy subjects with (n = 14) or without (n = 14) mild sleep complaints (poor and good sleepers, respectively). Repeated-measures ANOVA showed a significant interaction effect of diet × block × group (P = 0.008). In the poor sleepers, morning reaction time was significantly lower halfway through the task (block 2) and was higher at the end of the task (block 3) after the evening intake of α-lactalbumin than after the intake of placebo. The quadratic trends were significantly different between treatments (P = 0.05) but not in the good sleepers.

FIGURE 6. Mean (±SEM) changes in the number of errors during a continuous performance task after an overnight sleep following the evening intake of a tryptophan-rich α-lactalbumin diet (■) or a casein (placebo) diet (□) in healthy subjects with (n = 14) or without (n = 14) mild sleep complaints (poor and good, respectively). Repeated-measures ANOVA showed a significant interaction effect of diet × block × group (P = 0.008). In the poor sleepers, morning reaction time was significantly lower halfway through the task (block 2) and was higher at the end of the task (block 3) after the evening intake of α-lactalbumin than after the intake of placebo. The quadratic trends were significantly different between treatments (P = 0.05) but not in the good sleepers.

FIGURE 4. Mean (±SEM) changes in reaction time (RT) during a continuous performance task after an overnight sleep following the evening intake of a tryptophan-rich α-lactalbumin diet (■) or a casein (placebo) diet (□) in healthy subjects with (n = 14) or without (n = 14) mild sleep complaints (poor and good sleepers, respectively). Repeated-measures ANOVA showed a significant effect of block (P = 0.045) and a significant interaction effect of diet × block × group (P = 0.05). In the poor sleepers, morning RT was significantly lower halfway through the task (block 2) and was higher at the end of the task (block 3) after the evening intake of α-lactalbumin than after the intake of placebo. The quadratic trends were significantly different between treatments (P = 0.05) but not in the good sleepers.
in separate groups showed a near-significant effect of diet × block in poor sleepers ($P = 0.07$) and no significant effect in good sleepers. As shown in Figure 7, poor sleepers showed higher latency from blocks 1 + 2 to the last block (as a function of time-on-task) after the A-LAC diet as compared with after the placebo diet; this effect was not found in the good sleepers.

**DISCUSSION**

The present study investigated whether evening consumption of A-LAC increases plasma tryptophan availability for uptake into the brain and improves morning alertness and attention in subjects with sleep complaints. The results showed a large increase in tryptophan availability after A-LAC intake. Morning alertness improved modestly but significantly in all subjects and was accompanied by improved vigilance performance in subjects with mild sleep complaints.

**Evening dietary effect on Trp:LNAA**

Evening consumption of A-LAC containing 4.8 g/100 g Trp resulted in a 130% increase in the plasma Trp:LNAA as compared with placebo. This indicated that, after the A-LAC diet, more tryptophan was available for uptake into the brain (32). This rise in Trp:LNAA is considerably higher than the 48% increase that was previously found after consumption of A-LAC containing 1.7 g/100 g tryptophan (18) and the previously reported 20–45% increases after the consumption of foods such as carbohydrates (22). Although a 40–50% variation in plasma Trp:LNAA is thought to be sufficient to change available brain tryptophan and 5-HT synthesis and release (18, 20, 32–34), a 130% increase may cause an even larger increase in available brain tryptophan (and probably 5-HT) and, therefore may also result in a greater release of functionally active brain 5-HT and related behavior as previously suggested (18, 35). This certainly merits further investigation.

**Dietary effect on early morning sleepiness**

It was assumed that A-LAC would especially induce feelings of sleepiness in the evening in poor sleepers, leading, in turn, to improved sleep and reduced feelings of sleepiness in the morning. Contrary to our expectations, there was no effect on evening sleepiness. We found that poor sleepers were sleepier in the evening, regardless of dietary condition. In contrast, there was a significant dietary effect on sleepiness in the morning. Poor sleepers and good sleepers felt less sleepy and more alert in the morning after the consumption of A-LAC than after the consumption of placebo in the evening. This apparent contradictory effect of diet on evening compared with morning sleepiness may, however, not be surprising after all. The subjects had just finished a computer test before evening sleepiness was measured as part of another study. In addition, evening changes in feelings of sleepiness do not necessarily reflect the transitional phase between wakefulness and sleep onset and subsequent sleep progress, whereas early morning changes in sleepiness do. Hence, changes in morning scores on the Stanford’s Sleepiness Scale most likely indicate higher alertness due to improved sleep (28).

**Dietary effects on early morning performance**

There was a significant increase in RT as a function of task duration, which indicated successful task manipulation of vigilance. Generally, increased sleepiness after poor sleep significantly reduces vigilance performance as evidenced by an increase in RTs and an increase in the number of errors (4). Only in poor sleepers was RT significantly reduced halfway through the task and became higher at the end of the task after evening A-LAC consumption. Moreover, only in these subjects was there a modest but significant reduction in the number of false alarms across task performance.

The results also showed higher parietal P300 amplitudes during the morning task performance in all subjects after evening intake of A-LAC. Together with the reduction in morning sleepiness after A-LAC intake, this finding indicates a general increase in attention (5). Hence, an increase in parietal P300 amplitude is thought to reflect a rise in neural activity involved in attention that is controlled by the activation or arousal state of the subjects and its consequent drive to perform (5). Together with the behavioral findings, it appears as if such an increase in cognitive resources modestly improved task performance in poor sleepers. In addition, in these subjects, P300 latency increased across task performance after evening intake of A-LAC; this is thought to reflect slower cognitive evaluation processes and may suggest that subjects take more time to perform well.

**Are the dietary effects of A-LAC intake due to improved sleep?**

The remaining question is whether the effects of evening A-LAC intake on subsequent morning sleepiness and performance are mediated by improved sleep. This appears to be likely. First, the effects cannot be caused by higher plasma tryptophan availability in the morning. On the basis of previous studies, it is expected that food-induced increases in plasma Trp:LNAA in
humans remain for ≈4–5 h after intake (with peak scores occurring ≈3 h after intake), followed by a fast gradual return to baseline values (33, 36, 37). In addition, the highest increases in the present study were expected around bedtime and should have been sustained for only a few hours after midnight. Second, previous findings showed reduced sleep after evening tryptophan depletion and improved sleep after evening increases in tryptophan availability (9, 10, 13, 14). In addition, it was shown by Minet-Ringuet et al (25) that even the intake of A-LAC containing lower tryptophan concentrations than used in the present study was capable of reducing sleep disturbances after food deprivation in rats. Third, current dietary findings on reduced morning scores on the Stanford Sleepiness Scale further support the assumption that morning alertness improves as a result of better sleep and reduced sleep loss (28). Finally, the increases in parietal (P300) event-related brain activity indicate enhanced arousal and attention processing (5), which have been found to decrease particularly after poor sleep (4). In addition, Smulders (38) reported lower morning P300 amplitudes and reduced performance accuracy after sleep deprivation, as indicated by increased RTs in combination with a higher amount of errors.

**Conclusion**

The present study showed a significant 130% increase in the plasma Trp:LNAA 2 h after the evening intake of an A-LAC–enriched standard diet as compared with a placebo diet. This was accompanied by reduced sleepiness and higher task-related brain activity the following morning, which suggests improved alertness due to better sleep. Only in subjects with mild sleep complaints was this accompanied by modest but significantly improved vigilance performance. The current findings support the assumption that evening consumption of tryptophan-rich A-LAC may improve early morning performance indirectly by enhancing available brain tryptophan and subsequent sleep improvement. However, further evidence is needed, including EEG sleep measures, in subjects with more severe sleep complaints. For instance, beneficial effects on morning performance may be mediated by reduced sleep onset latency or changes in rapid eye movement latency, because these are particularly affected by acute tryptophan depletion (10) or by 1-tryptophan (10).

We express our appreciation to Cees van Leeuwen for his medical consulting assistance. CRM designed the study and was responsible for the overall data analysis and the writing of the manuscript. LMI participated in the study design and was responsible for the EEG-ERP methodology and data acquisition. LMI, JHCM, MHM, and NR participated in conducting the study and in collecting and analyzing the data. NEPD was responsible for the plasma amino acid analysis. All authors reviewed the study protocol and the manuscript. None of the authors had any conflicts of interest.

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Genetic and environmental contributions to serum retinol and α-tocopherol concentrations: the Stanislas Family Study

Sonia Gueguen, Pierre Leroy, René Gueguen, Gérard Siest, Sophie Visvikis, and Bernard Herbeth

ABSTRACT

Background: Although numerous environmental factors are documented to influence serum retinol and α-tocopherol concentrations, little is known about the genetic versus the environmental contributions to variations in these traits.

Objective: The aim of this study was to estimate additive genetic heritability and household effects for serum retinol and α-tocopherol concentrations in a variance component analysis.

Design: In a sample of 387 French families, information on serum retinol and α-tocopherol concentrations, usual dietary intake, lifestyle, and serum lipid profiles and related polymorphisms (apolipoprotein E, apolipoprotein C-III, apolipoprotein B, cholesteryl ester transfer protein, and lipoprotein lipase) was obtained.

Results: For serum retinol—after adjustment for sex, age, body mass index, alcohol consumption, oral contraceptive use, and serum albumin, triacylglycerol, and apolipoprotein A-I concentrations—additive genetic effects and shared common environment contributed 30.5% and 14.2% of the total variance, respectively. For serum α-tocopherol, ≈22.1% of the total variance was due to the additive effects of genes and 18.7% to those of household environment, after adjustment for the covariates sex, age, vitamin E intake, oral contraceptive use, and cholesterol, triacylglycerol, and apolipoprotein A-I concentrations. For both vitamins, the influence of measured polymorphisms was not significant. Moreover, heritability and household effect estimates were not significantly different between the 4 classes of relatives and did not vary significantly when families shared more meals at home.


KEY WORDS Retinol, α-tocopherol, family resemblance, genetics, household environment

INTRODUCTION

Developing statistical methods to analyze data collected from nuclear families allows investigation of the traits that underlie the strong influence of family history on the risk of various chronic diseases, such as cancer, osteoporosis, and atherosclerosis, and related cardiovascular complications. The identification of heritability should be a prerequisite for the search for quantitative trait loci affecting such traits and thereby modulating the risk of disease. Such investigations, however, must account for the fact that these intermediate traits themselves are multifactorial in nature, being influenced by both genes and environmental factors. Strong evidence indicates genetic familial influences on bone mineralization (1), body mass index (2), dyslipidemia (3), glucose intolerance (3), hypertension (3), hemostatic factors (4), platelet aggregation (5), total antioxidant activity (6), and vitamin D concentrations (7). However, data regarding the genetic epidemiology of circulating indexes of retinol and α-tocopherol status are lacking.

Mutations in genes encoding key proteins, such as retinol-binding protein (RBP) (8)—the main carrier for vitamin A—or prealbumin (9), which is known to form a complex with RBP in plasma, have been identified. Such genetic defects induce severe biochemical vitamin A deficiency and low concentrations of plasma retinol and RBP.

Concerning vitamin E, mutations in the α-tocopherol transfer protein (α-TTR) gene have been detected in patients with low plasma α-tocopherol and ataxia with isolated vitamin E deficiency (10, 11). α-TTR is a liver protein responsible for the selective retention of α-tocopherol from dietary vitamin E and for its transfer into nascent VLDL. This key phase is the major determinant of plasma α-tocopherol concentrations in humans (12).

Because of the particular metabolism of these 2 fat-soluble vitamins (13, 14), genes whose products affect lipoprotein metabolism, eg, apolipoproteins (apos), enzymes, and receptors, particularly in response to dietary change, should be taken into account (15, 16). However, few studies have investigated this possibility (17). The present study aimed at estimating additive genetic heritability and household effects of serum retinol and α-tocopherol concentrations in a variance component analysis, with adjustment for the influence of known environmental covariates and polymorphisms of apo E, apo C-III, and apo B; cholesteryl ester transfer protein (CETP); and lipoprotein lipase (LPL) known to affect lipid and lipoprotein metabolism.

1 From the INSERM U525, Nancy, France (SG, GS, SV, and BH); the Faculté de Pharmacie, Nancy, France (PL); and the Centre de Médecine Préventive, Vandœuvre-lès-Nancy, France (RG and GS).
2 Supported by grants from The Centre d’Etude et d’Information sur les Vitamines and Kellogg’s PA.
3 Address reprint requests to B Herbeth, INSERM U525, Centre de Médecine Préventive, 2 rue Jacques Pariset, 54500 Vandœuvre-lès-Nancy, France. E-mail: bernard.herbeth@cmp.u-nancy.fr
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SUBJECTS AND METHODS

Subjects and study design

This work is part of the Stanislas Family Study, a 10-y longitudinal follow-up study conducted since 1994 in 1006 families selected at the Center for Preventive Medicine of Vandoeuvre-lès-Nancy (France) who were free of chronic or acute disease that could influence nutritional status (18). In this article, we present data from the first examination (1994–1995), which was obtained from a random subsample of 383 families composed of 2 parents aged 28–59 y and at least one child between 6 and 24 y of age (n = 1487). All subjects underwent a complete medical examination, including weight and height measurements. Data on alcohol consumption, smoking status, and drug use (especially oral contraceptives, lipid-lowering agents, and vitamin supplements) were collected through validated questionnaires under the supervision of trained nurses. The research protocol was approved by the “Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Lorraine” and each subject gave written informed consent.

Dietary record

Dietary intake was assessed with a 3-d dietary record (19), which was completed during 2 weekdays and a 1 weekend day assigned at random for each family. All subjects received guidelines from a dietician on the procedures for completing the dietary record and measuring food portions. For young children, the 3-d diary was filled in by the mother and the child together. One week later, the 3-d record was checked and completed by the dietician with the use of colored photographs of foods, each with 3 different portion sizes. Macronutrient and micronutrient intakes were estimated with an updated computerized version of the Répertoire Général des Aliments (20). Vitamin A intakes were expressed in retinol activity equivalents and estimated as preformed retinol + β-carotene equivalents/12; β-carotene equivalents were the sum of β-carotene and one-half of the amounts of α-carotene, γ-carotene, and cryptoxanthin (21). Vitamin E intakes, expressed in α-tocopherol equivalents, were calculated from measurements of α-tocopherol and other compounds with vitamin E activity by using recommended conversion factors (20): α-tocopherol + β-tocopherol/2.5 + γ-tocopherol/10 + δ-tocopherol/100 + α-tocotrienol/3.3 + γ-tocotrienol/20 + δ-tocotrienol/100.

In addition, families had to report the number of meals shared at home with all other relatives during the week. An index of shared meals was computed for each family as the average number of meals shared at home by 2 persons in the family in a week. When the analysis of variance was significant, a Tukey-Kramer test was used to detect which groups were statistically different from each other.

Because of the design of the Stanislas Family Study, the 3-d food consumption diary was filled during the week after the first examination, including weight and height measurements. Data on alcohol consumption, smoking status, and drug use (especially oral contraceptives, lipid-lowering agents, and vitamin supplements) were collected through validated questionnaires under the supervision of trained nurses. The research protocol was approved by the “Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Lorraine” and each subject gave written informed consent.

Biological measurements

Blood samples were collected after the subjects fasted overnight. Serum albumin, cholesterol, and triacylglycerol concentrations were measured by using commercially available kits (Merck, Darmstadt, Germany) on AU5021 apparatus (Olympus; Merck). Serum apo A-I and B were determined by immunonephelometry with a Behring Nephelometer Analyzer and Behring reagents (Rueil-Malmaison, France). Frozen aliquots of serum were stored in the bio bank of the Centre de Médecine Préventive (Vandoeuvre les Nancy, France).

A reversed-phase HPLC method was used for the simultaneous determination of serum retinol and α-tocopherol as adapted from Rudy et al (23). Analytes were isolated by liquid-liquid extraction, concentrated by evaporation, and then chromatographed on an C18, 15 cm × 4.6 mm ODS 5-μm column (Beckman Instruments, Gagny, France), with the use of a water/methanol–ethyl acetate/isopropanol solvent system. Retinol and α-tocopherol are spectrophotometrically detected at 325 and 292 nm, respectively. Standards (retinol and α-tocopherol) and their respective internal standards (retinol acetate and α-tocopherol acetate) were purchased from Fluka Chemie AG (Buchs, Switzerland) and solvents (HPLC grade) from Merck. Between-run variability was controlled by using a frozen control serum that was measured in duplicate within each of the 45 runs of analysis. The interassay CVs were 8.1% for retinol and 10.7% for α-tocopherol.

DNA polymorphism determination

Genomic DNA was extracted from peripheral blood samples by the salting out method (24). The genotypes of apo E Cys112Arg and Arg118Cys, apo B Thr71Ile, apo C-III C(−482)T, apo C-III C1100T, CETP Ile405Val, and LPL Ser447Term were determined with a multiplex assay that was described previously by Cheng et al (25).

Statistical analysis

Individual statistical analyses were performed by using the SAS software package version 8.01 (SAS Institute Inc, Cary, NC). Triacylglycerol concentrations were log10-transformed in the analyses to improve normality. A chi-square test was performed to determine whether genotype frequencies were in Hardy-Weinberg equilibrium. Before individual and familial statistical analyses, serum retinol and α-tocopherol concentrations were adjusted for the effect of between-run variation. Briefly, concentrations were regressed on mean values of the frozen pool of serum measured in duplicate in each series of dosage; the variable used was the sum of residual + crude mean of the overall sample.

For continuous variables, an analysis of variance was performed for characteristic differences between the 4 groups of relatives. When the analysis of variance was significant, a Tukey-Kramer test was used to detect which groups were statistically different from each other. The significance of differences among the groups for the categorical variables was analyzed by using the chi-square test or the Fisher’s exact test when cells had expected counts of <5.
In the overall sample, stepwise multiple regression analysis was carried out to select significant covariates \((P \leq 0.05)\) among lifestyle factors, diet intake, related biological analytes, and genetic variants. Then, regression coefficients were computed for the overall sample and for the 4 sex-by-generation groups (fathers, mothers, sons, and daughters). In addition, the significance of differences in regression coefficients between fathers, mothers, sons, and daughters was assessed by testing interaction terms between each covariate and the 4 groups in the overall sample. Because individuals within a family are not independent, statistical analyses were based on the estimating equation technique by using the SAS GENMOD procedure with a repeated statement.

Intrafamilial correlations were estimated by using maximum likelihood techniques \((26)\) with and without adjustment for covariates. This statistical program allowed adjustment for covariates within models, simultaneously and separately for fathers, mothers, sons, and daughters. The significance of various familial correlations or sex and generation differences in correlations was tested by using the log-likelihood ratio test. Correlations were computed under 4 sets of hypotheses: sex effects on correlations for parents and children (general model), sex effects only for children (submodel 1), sex effects only for parents (submodel 2), and no sex effects at all (submodel 3).

Variance component analysis was applied to assess the relative contributions of genetic factors, common household factors, and individual specific environment in family aggregation of serum retinol and \(\alpha\)-tocopherol concentrations. The variable used to estimate the variance component was adjusted for significant covariates—separately for fathers, mothers, sons, and daughters—and was standardized to zero mean and unit variance within each sex-by-generation group. The analysis was conducted by using a multivariate normal model for pedigree analysis as described by Lange et al \((27, 28)\) with FISHER software, which also performed tests of goodness-of-fit of the underlying multinormal distribution. The general model assumed that the studied trait was the result of the sum of 3 independent random components: a polygenic component \((G)\) representing additive genetic factors, household factors common to individuals within a family \((H)\), and unmeasured environmental factors particular to an individual, including measurement error \((E)\). These 3 components were assumed to be normally distributed with a mean equal to 0 and a variance equal to \(\sigma^2_G\), \(\sigma^2_H\), and \(\sigma^2_E\), respectively.

The hypothesis of no polygenic component or no household effect was checked by comparing a model including \(\sigma^2_G\), \(\sigma^2_H\), and \(\sigma^2_E\) with a model including only \(\sigma^2_H\) and \(\sigma^2_E\), or \(\sigma^2_G\) and \(\sigma^2_E\), respectively. In addition, possible effects of covariates on these variance components were tested, such as age, sex, and frequency of sharing meals (families were categorized in 2 groups of similar size: <12 (\(n = 199\)) or \(\geq 12 (n = 184)\) meals shared/wk).

Comparison of nested models was based on the likelihood ratio criteria. Eventually, the best parsimonious model was selected. The contribution (as a percentage) of the 3 components—additive genetic factors (heritability), household factors, and residual environmental—to residual phenotypic variance (after adjustment for covariates) was deduced.

RESULTS

Descriptive characteristics and daily intakes of energy, macronutrients, and vitamins E and A for the 4 sex-by-generation groups are summarized in Table 1. Vitamin A and vitamin E intakes were significantly higher in fathers than in daughters; the mother’s and son’s intakes were intermediate. When expressed per kJ, the nutritional densities of vitamin A were significantly higher in parents than in children. For vitamin E, nutritional density was significantly higher in mothers than in the 3 other groups.

Serum retinol and \(\alpha\)-tocopherol concentrations, related biological analytes, and allelic frequencies of selected polymorphisms are presented in Table 2. The apo E, apo B, apo C-III, CETP, and LPL polymorphism distribution did not significantly deviate from Hardy-Weinberg equilibrium. Retinol concentrations in fathers were significantly higher than in children; mothers had intermediate concentrations. \(\alpha\)-Tocopherol concentrations were significantly different between the 4 groups: fathers had the highest concentrations and sons the lowest. Others characteristics of Tables 1 and 2 were in accordance with the values found in apparently healthy individuals of the same age and sex.

Crude and adjusted mean serum retinol and \(\alpha\)-tocopherol concentrations are shown according to sex and age classes \((5–9, 10–12.4, 12.5–14, 15–17.4, 17.5–19, 20–24, 25–34, 35–39, 40–44, 45–49, and 50–59 y)\) in Figures 1 and 2. Values were adjusted for the covariates in Tables 3 and 4.

Crude values for serum retinol concentrations increased with age in both boys and girls from 10 to 24 y of age \((P \leq 0.001)\); the difference between sexes was not significant. In men and women, serum retinol concentrations did not vary significantly with age. Between 25 and 59 y, serum retinol concentrations were significantly higher in males than in females \((P \leq 0.001)\). After adjustment for covariates, age-related variations were of lower range.

From 10 to 14 y, crude values for \(\alpha\)-tocopherol concentrations significantly decreased and then increased from 15 to 59 y in both males and females \((P \leq 0.001)\). Before the age of 24 y, females had higher mean values than did males \((P \leq 0.05)\); after the age of 24 y the opposite was observed \((P \leq 0.01)\). As for serum retinol concentrations, age-related variations of adjusted \(\alpha\)-tocopherol concentrations were weaker and the interaction of age and sex was not significant.

Predictors of serum retinol concentrations in parents and offspring are presented in Table 3. The proportion of phenotypic variance accounted for by the measured covariates ranged from 11.3% (in fathers) to 48.8% (in daughters). In the overall sample, females had significantly lower concentrations than did males, and oral contraceptive use, alcohol consumption, and serum albumin and apo AI concentrations were significantly and positively related to serum retinol concentrations; interactions between each covariate and the 4 subgroups were not statistically significant. On the other hand, statistically significant interactions were observed for age (positive regression coefficients being significant only in offspring and significantly higher in sons than in daughters), serum triacylglycerol (positive regression coefficients being significant in the 4 subgroups and significantly lower in sons than in parents and daughters), and BMI (regression coefficients being significant only in fathers and offspring).

Predictors of serum \(\alpha\)-tocopherol concentrations in parents and offspring are presented in Table 4. The proportion of phenotypic variance accounted for by the measured covariates ranged from 40.4% (in sons) to 52.7% (in fathers). In the overall sample, females had significantly higher concentrations than did...
### TABLE 1
Descriptive characteristics according to the 4 sex-by-generation groups: lifestyle factors and diet intake

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Fathers (n = 383)</th>
<th>Mothers (n = 383)</th>
<th>Sons (n = 341)</th>
<th>Daughters (n = 380)</th>
<th>p²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>42.2 ± 4.7a,b</td>
<td>40.3 ± 4.4b,c</td>
<td>14.0 ± 3.4c</td>
<td>14.3 ± 3.9c</td>
<td>0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.5 ± 3.2a</td>
<td>23.6 ± 3.9b,c</td>
<td>19.3 ± 3.2c</td>
<td>19.6 ± 3.2c</td>
<td>0.001</td>
</tr>
<tr>
<td>Alcohol consumption (g/d)</td>
<td>22.0 ± 22.4a</td>
<td>5.9 ± 9.7b,c</td>
<td>0.9 ± 3.3c</td>
<td>0.6 ± 2.4c</td>
<td>0.001</td>
</tr>
<tr>
<td>Tobacco consumption (g/d)</td>
<td>5.0 ± 9.4a</td>
<td>2.5 ± 6.4b,c</td>
<td>0.4 ± 1.9c</td>
<td>0.7 ± 2.8c</td>
<td>0.001</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>30.8a</td>
<td>19.5b,c</td>
<td>4.4c</td>
<td>7.4c</td>
<td>0.001</td>
</tr>
<tr>
<td>Vitamin E supplement use (%)</td>
<td>0.8a</td>
<td>0.0b</td>
<td>0.0c</td>
<td>0.0d</td>
<td>0.157</td>
</tr>
<tr>
<td>Oral contraceptive use (%)</td>
<td>—</td>
<td>21.1¹</td>
<td>—</td>
<td>15.2b</td>
<td>0.035</td>
</tr>
<tr>
<td>Menopause status (%)</td>
<td>—</td>
<td>2.1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Estrogen or progestagen use (%)</td>
<td>—</td>
<td>9.2a</td>
<td>—</td>
<td>0.3b</td>
<td>0.001</td>
</tr>
<tr>
<td>Hypolipidemic drug use (%)</td>
<td>7.6a</td>
<td>2.3b</td>
<td>0.8c</td>
<td>0.3c</td>
<td>0.001</td>
</tr>
<tr>
<td>Diet intake</td>
<td>—</td>
<td>2.1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

| | | | | | |
| Energy (MJ) | 9.7 ± 2.19a | 7.4 ± 1.79b | 10.11 ± 2.36c | 7.98 ± 1.60d | 0.001 |
| Protein (% of energy) | 17.9 ± 2.9a | 17.9 ± 3.3a | 15.6 ± 2.4b | 15.6 ± 2.8b | 0.001 |
| Carbohydrate (% of energy) | 44.9 ± 6.7a | 44.6 ± 6.7a | 48.9 ± 5.6b | 48.0 ± 5.6b | 0.001 |
| Fat (% of energy) | 37.2 ± 5.8a | 37.4 ± 5.7a | 35.4 ± 4.9b | 36.4 ± 5.1b | 0.001 |
| Vitamin A (µg/d) | 1101 ± 1103a | 972 ± 1195b,c | 820 ± 880b,c | 675 ± 583c | 0.001 |
| Vitamin E (µg/d) | 117 ± 127a | 135 ± 168b | 83 ± 92b | 85 ± 72b | 0.001 |
| Vitamin E (µg/kJ) | 0.86 ± 0.35a | 1.00 ± 0.43b | 0.81 ± 0.35c | 0.88 ± 0.43d | 0.001 |

¹ Means in the same row with different superscript letters are significantly different, P ≤ 0.05 (Tukey-Kramer test).
² For differences between the 4 groups tested with ANOVA, chi-square test, or Fisher’s exact test (for oral contraceptive and estrogen or progestagen use, differences were tested between mothers and daughters).
³ x ± SD (all such values).
⁴ Expressed as a percentage of nonalcohol energy intakes.
⁵ Retinol activity equivalents.
⁶ Nutritional density: vitamin intakes (µg/d)/energy intakes (kJ/d).
⁷ α-Tocopherol equivalents.

### TABLE 2
Descriptive characteristics according to the 4 sex-by-generation groups: serum vitamin concentrations, related biological covariates, and genetic variants

<table>
<thead>
<tr>
<th>Vitamin concentration</th>
<th>Fathers (n = 383)</th>
<th>Mothers (n = 383)</th>
<th>Sons (n = 341)</th>
<th>Daughters (n = 380)</th>
<th>p²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinol (µmol/L)</td>
<td>2.64 ± 0.55a</td>
<td>2.09 ± 0.52b</td>
<td>1.77 ± 0.43c</td>
<td>1.80 ± 0.54d</td>
<td>0.001</td>
</tr>
<tr>
<td>α-tocopherol (µmol/L)</td>
<td>31.1 ± 7.3a</td>
<td>28.6 ± 5.8b</td>
<td>21.9 ± 5.0c</td>
<td>23.5 ± 4.5d</td>
<td>0.001</td>
</tr>
</tbody>
</table>

| Related biological analytes | | | | |
| Total cholesterol (mmol/L)⁵ | 6.00 ± 1.05a | 5.54 ± 0.91b | 4.56 ± 0.86c | 4.83 ± 0.80d | 0.001 |
| Triacylglycerol (mmol/L)⁵ | 1.17b (0.67–2.03) | 0.78b (0.49–1.24) | 0.66b (0.42–1.02) | 0.73b (0.48–1.12) | 0.001 |
| Apolipoprotein A-I (g/L)⁵ | 1.54 ± 0.24d | 1.70 ± 0.26b | 1.45 ± 0.24d | 1.53 ± 0.22d | 0.001 |
| Apolipoprotein B (g/L)⁵ | 1.15 ± 0.26b | 0.98 ± 0.22b | 0.79 ± 0.20b | 0.83 ± 0.18b | 0.001 |
| Albumin (g/L)⁵ | 45.7 ± 1.9b | 44.3 ± 2.0b | 46.4 ± 2.0b | 45.9 ± 2.1a | 0.001 |

| Allelic frequencies (%) | | | | |
| Apo E2 | 6.8 | 9.3 | 8.9 | 8.7 | 0.763 |
| Apo E4 | 13.1 | 13.8 | 10.8 | 14.2 | 0.273 |
| Apo B Thr71Ile | 33.5 | 31.8 | 34.0 | 32.5 | 0.820 |
| Apo C-III C(−482)T | 27.3 | 27.0 | 26.5 | 26.1 | 0.962 |
| Apo C-III C1100T | 26.5 | 27.4 | 28.8 | 26.4 | 0.695 |
| LPL Ser447term | 12.5 | 11.8 | 11.1 | 12.3 | 0.856 |
| CETP Ile405Val | 32.0 | 28.6 | 32.2 | 30.3 | 0.370 |

¹ Means in the same row with different superscript letters are significantly different, P ≤ 0.05 (Tukey-Kramer test).
² For differences between the 4 groups tested with ANOVA or chi-square test.
³ Arithmetic x ± SD.
⁴ Test on log10-transformed values.
⁵ Geometric x; range of 1 SD in parentheses.
males, and age, vitamin E intake, and cholesterol and apo A-I concentrations were significantly and positively associated with serum α-tocopherol concentrations; interactions between each covariate and the 4 subgroups were not statistically significant. On the other hand, statistically significant interactions were observed for serum triacylglycerol (positive regression coefficients being significant in the 4 subgroups and significantly higher in parents than in children), vitamin E supplement use (positive regression coefficients being significant only in fathers), and oral contraceptive use (negative regression coefficient being significant only in mothers).

The polymorphisms of apo E, apo C-III, apo B, CETP, and LPL were not significantly related to serum retinol concentrations when either crude or adjusted values for the covariates listed in Table 3 were used (data not shown). Apo E ε4 and apo C-III 1100C alleles were significantly associated with crude values of serum α-tocopherol concentrations \( P \leq 0.01 \), whereas a significant inverse association was noted for the apo E ε2 allele \( P \leq 0.01 \). However, after adjustment for the covariates listed in Table 4, no polymorphism was significantly related to serum α-tocopherol concentrations (data not shown).

The patterns of family correlations for serum retinol and α-tocopherol concentrations are given in Tables 5 and 6 for crude and adjusted values. For both serum retinol and α-tocopherol concentrations, the general model that took into account covariates was the most parsimonious. When adjusted values were used, the 2 variables showed significant familial correlations by rejecting the hypothesis that no familial resemblance exists within the families (all \( P \leq 0.001 \); data not shown). Significant correlations \( P \leq 0.01 \) and \( P \leq 0.001 \) were found for all the various pairs of relatives, except for son-son serum α-tocopherol correlations.

The hypothesis of no difference in correlations for father-offspring and mother-offspring (FS = MS and FD = MD), but with different correlations according to child sex, was tested with submodel 1. Submodel 2 assumed no effect of child sex on family correlations (FS = FD, MS = MD and SS = SD = DD). Submodel 3 hypothesized no effect of sex on family correlations (FS = MS = MD = FD and SS = SD = DD). For both serum retinol and α-tocopherol concentrations, the most adequate parsimonious model was submodel 3. Spouse, parent-offspring, and offspring-offspring correlation coefficients were 0.129, 0.289, and 0.368 for serum retinol, respectively, and 0.198, 0.307, and 0.243 for serum α-tocopherol, respectively.

Quantitative genetic analyses were performed for serum retinol concentrations after adjustment for the covariates previously described in Table 3. The components of variance attributable to additive genetic effects, shared household effects, and residual environmental factors (including assay imprecision) are shown in Table 7. The full model 1, including the 3 components, cannot be reduced to a simpler model (submodels M2, M3, and M4 were rejected by log-likelihood tests). The proportion of phenotypic
variability accounted for by household was smaller (about half)  

than that accounted for by genetic effects: 30.5% compared with

14.2%. Component attributable to residual environment factors  

represented more than half of the total variance (54.8%). More

complex models hypothesizing effects of sex and generation on  

the 3 variance components (models 5 to 7) or an effect of the  

number of meals shared together at home (model 8) did not

significantly improve the log-likelihood function.

**TABLE 3**  

Predictors of serum retinol concentrations in multiple regression analysis in the whole sample and in the 4 groups separately

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Fathers (n = 383)</th>
<th>Mothers (n = 383)</th>
<th>Sons (n = 341)</th>
<th>Daughters (n = 380)</th>
<th>Overall group (n = 1487)</th>
<th>P for interaction²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>−0.001 (0.000)⁻اته</td>
<td>0.008 (0.0016)⁻اته</td>
<td>0.039 (0.0016)⁻اته</td>
<td>0.014 (0.2378)⁻اته</td>
<td>0.009 (0.2450)⁻اته</td>
<td>0.002</td>
</tr>
<tr>
<td>Serum triacylglycerol (mmol/L)</td>
<td>0.474 (0.0541)⁻اته</td>
<td>0.767 (0.1535)⁻اته</td>
<td>0.246 (0.0127)⁻اته</td>
<td>0.565 (0.0707)⁻اته</td>
<td>0.611 (0.0963)⁻اته</td>
<td>0.008</td>
</tr>
<tr>
<td>Oral contraceptive use vs no use</td>
<td>—</td>
<td>0.571 (0.1833)⁻اته</td>
<td>—</td>
<td>0.686 (0.1499)⁻اته</td>
<td>0.677 (0.0458)⁻اته</td>
<td>0.259</td>
</tr>
<tr>
<td>Female sex vs male sex</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Alcohol consumption (g/d)</td>
<td>0.003 (0.0158)⁻اته</td>
<td>0.006 (0.0144)⁻اته</td>
<td>0.017 (0.0142)⁻اته</td>
<td>0.008 (0.0016)⁻اته</td>
<td>0.006 (0.0159)⁻اته</td>
<td>0.236</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.020 (0.0134)⁻اته</td>
<td>0.005 (0.0001)⁻اته</td>
<td>0.035 (0.0318)⁻اته</td>
<td>0.022 (0.0091)⁻اته</td>
<td>0.025 (0.0154)⁻اته</td>
<td>0.009</td>
</tr>
<tr>
<td>Serum albumin (g/L)</td>
<td>0.037 (0.0190)⁻اته</td>
<td>0.021 (0.0097)⁻اته</td>
<td>0.029 (0.0155)⁻اته</td>
<td>0.005 (0.014)⁻اته</td>
<td>0.034 (0.0122)⁻اته</td>
<td>0.349</td>
</tr>
<tr>
<td>Serum apolipoprotein A-1 (g/L)</td>
<td>0.259 (0.0109)⁻اته</td>
<td>0.236 (0.0118)⁻اته</td>
<td>0.166 (0.0073)⁻اته</td>
<td>0.341 (0.0168)⁻اته</td>
<td>0.179 (0.0042)⁻اته</td>
<td>0.602</td>
</tr>
<tr>
<td>Intercept</td>
<td>−0.034</td>
<td>0.222</td>
<td>−0.997</td>
<td>0.448</td>
<td>−0.499</td>
<td></td>
</tr>
<tr>
<td>Global R²</td>
<td>0.113</td>
<td>0.375</td>
<td>0.383</td>
<td>0.488</td>
<td>0.483</td>
<td></td>
</tr>
</tbody>
</table>

⁻¹ All values are regression coefficients; values in parentheses represent partial R². Regression coefficients in the same row with different superscript letters are significantly different, P ≤ 0.05.

² Test for group × covariate interactions.

³ P > 0.10 (compared with zero).

⁴ P ≤ 0.001 (compared with zero).

⁵ P ≤ 0.05 (compared with zero).

⁶ Log-transformed values.

⁷ P ≤ 0.01 (compared with zero).

⁸ P ≤ 0.10 (compared with zero).
Quantitative genetic analyses for serum α-tocopherol concentrations after adjustment for the covariates described in Table 4 are shown in Table 8. As for serum retinol concentrations, the model giving the best description of the variance decomposition included the 3 components (model 1); heritability was lower and close to that attributable to household effects (22.1% compared with 18.7%), and the component attributable to residual environmental factors represented more than half of the total variance (59.1%). As for serum retinol concentrations, taking into account the relative level and the number of meals taken together in 2 class (<12 or ≥12 meals shared per week) did not improve significantly the likelihood function (models 5 to 8).

**DISCUSSION**

No data are available regarding the inheritance of serum concentrations of retinol and α-tocopherol, apart from results about genetic mutations of key enzymes or proteins involved in the regulation of these 2 fat-soluble vitamins in serum (8, 11). In our study, for both

### TABLE 4
Predictors of serum α-tocopherol concentrations in multiple regression analysis in the whole sample and in the 4 groups separately

<table>
<thead>
<tr>
<th></th>
<th>Fathers (n = 383)</th>
<th>Mothers (n = 383)</th>
<th>Sons (n = 341)</th>
<th>Daughters (n = 380)</th>
<th>Overall group (n = 1487)</th>
<th>P for interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum cholesterol (mmol/L)</td>
<td>2.80 (0.3708)c</td>
<td>3.31 (0.4452)c</td>
<td>3.04 (0.3742)c</td>
<td>3.13 (0.3968)c</td>
<td>3.21 (0.5352)c</td>
<td>0.685</td>
</tr>
<tr>
<td>Serum triacylglycerol (mmol/L)c</td>
<td>13.04 (0.1180)c</td>
<td>7.24 (0.0315)c</td>
<td>3.57 (0.0099)c</td>
<td>3.91 (0.0144)c</td>
<td>7.48 (0.0412)c</td>
<td>0.001</td>
</tr>
<tr>
<td>Age (y)</td>
<td>0.088 (0.0034)c</td>
<td>-0.001 (0.0037)c</td>
<td>0.056 (0.0000)c</td>
<td>-0.022 (0.0017)c</td>
<td>0.081 (0.0238)c</td>
<td>0.457</td>
</tr>
<tr>
<td>Serum apolipoprotein A-I (g/L)</td>
<td>3.37 (0.0093)c</td>
<td>2.76 (0.0065)c</td>
<td>3.37 (0.0192)c</td>
<td>2.35 (0.0078)c</td>
<td>2.71 (0.0052)c</td>
<td>0.814</td>
</tr>
<tr>
<td>Oral contraceptive use vs no use</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Vitamin E supplement vs none</td>
<td>13.38 (0.0247)c</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Female sex vs male</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Vitamin E intake (mg/d)</td>
<td>0.067 (0.0011)c</td>
<td>0.172 (0.0104)c</td>
<td>0.023 (0.0003)c</td>
<td>0.047 (0.0016)c</td>
<td>0.059 (0.0008)c</td>
<td>0.264</td>
</tr>
<tr>
<td>Intercept</td>
<td>3.78</td>
<td>5.60</td>
<td>2.85</td>
<td>5.44</td>
<td>3.14</td>
<td>—</td>
</tr>
<tr>
<td>Global R²</td>
<td>0.527</td>
<td>0.525</td>
<td>0.404</td>
<td>0.427</td>
<td>0.619</td>
<td>—</td>
</tr>
</tbody>
</table>

1 All values are regression coefficients; values in parentheses represent partial R². Regression coefficients in the same row with different superscript letters are significantly different, P ≤ 0.05.

2 Test for group × covariate interactions.

3 P ≤ 0.001.

4 Log-transformed values.

5 P ≤ 0.01.

6 P > 0.10.

7 P ≤ 0.05.

### TABLE 5
Estimates of familial correlations for serum retinol concentrations

<table>
<thead>
<tr>
<th></th>
<th>General model 1</th>
<th>General model 2</th>
<th>Submodel 1</th>
<th>Submodel 2</th>
<th>Submodel 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FM</td>
<td>0.137 ± 0.050²</td>
<td>0.129 ± 0.050²</td>
<td>0.129 ± 0.050²</td>
<td>0.129 ± 0.050²</td>
<td>0.129 ± 0.050²</td>
</tr>
<tr>
<td>FS</td>
<td>0.187 ± 0.057²</td>
<td>0.168 ± 0.056²</td>
<td>0.280 ± 0.037²</td>
<td>0.236 ± 0.040²</td>
<td>0.289 ± 0.029²</td>
</tr>
<tr>
<td>FD</td>
<td>0.239 ± 0.049²</td>
<td>0.285 ± 0.049²</td>
<td>0.296 ± 0.037²</td>
<td>[0.236]</td>
<td>[0.289]</td>
</tr>
<tr>
<td>MS</td>
<td>0.306 ± 0.050²</td>
<td>0.376 ± 0.046²</td>
<td>[0.280]</td>
<td>0.341 ± 0.037²</td>
<td>[0.289]</td>
</tr>
<tr>
<td>MD</td>
<td>0.230 ± 0.052²</td>
<td>0.305 ± 0.052²</td>
<td>[0.296]</td>
<td>[0.341]</td>
<td>[0.289]</td>
</tr>
<tr>
<td>SS</td>
<td>0.504 ± 0.067²</td>
<td>0.369 ± 0.082²</td>
<td>0.362 ± 0.084²</td>
<td>0.368 ± 0.046²</td>
<td>0.368 ± 0.046²</td>
</tr>
<tr>
<td>SD</td>
<td>0.212 ± 0.069²</td>
<td>0.321 ± 0.070²</td>
<td>0.323 ± 0.070²</td>
<td>[0.368]</td>
<td>[0.368]</td>
</tr>
<tr>
<td>DD</td>
<td>0.242 ± 0.098²</td>
<td>0.433 ± 0.074²</td>
<td>0.434 ± 0.074²</td>
<td>[0.368]</td>
<td>[0.368]</td>
</tr>
<tr>
<td>−Log Lf</td>
<td>1052.93</td>
<td>715.41</td>
<td>719.60</td>
<td>718.27</td>
<td>720.27</td>
</tr>
<tr>
<td>Alternate model</td>
<td>—</td>
<td>General model 1</td>
<td>General model 2</td>
<td>General model 2</td>
<td>General model 2</td>
</tr>
<tr>
<td>χ² (df)</td>
<td>—</td>
<td>675.04 (28)</td>
<td>8.38 (2)</td>
<td>5.72 (4)</td>
<td>9.72 (5)</td>
</tr>
<tr>
<td>P</td>
<td>—</td>
<td>≤0.001</td>
<td>≤0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

1 General models estimated all 8 correlations: GMI used crude values and GM2 used adjusted values for age, oral contraceptive use, BMI, alcohol intake, and albumin, triacylglycerol, and apolipoprotein A-I concentrations. The submodels used adjusted values for the above-listed covariates: SMI (no sex effect on parents correlations: FS = MS and FD = MD), SM2 (no sex effect on offspring correlations: FS = FD, MS = MD and SS = SD = DD), and SM3 (no sex effect on parents or offspring correlations: FS = MS = MD = FD and SS = SD = DD). Values in brackets were constrained to be equal to a preceding value according to the hypotheses of the submodel. FM, father-mother pair; FS, father-son pair; FD, father-daughter pair; MS, mother-son pair; MD, mother-daughter pair; SS, son-son pair; SD, son-daughter pair; DD, daughter-daughter pair.

2 Correlation coefficient ± SE (all such values).

3 P ≤ 0.01 (compared with zero).

4 P ≤ 0.001 (compared with zero).

5 Logarithm of likelihood function.
TABLE 6
Estimates of familial correlations for serum α-tocopherol concentration

<table>
<thead>
<tr>
<th></th>
<th>General model 1</th>
<th>General model 2</th>
<th>Submodel 1</th>
<th>Submodel 2</th>
<th>Submodel 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FM</td>
<td>0.060 ± 0.050&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.198 ± 0.049&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.198 ± 0.049&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.198 ± 0.049&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.198 ± 0.049&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>FS</td>
<td>0.234 ± 0.049&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.309 ± 0.043&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.308 ± 0.034&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.286 ± 0.037&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.307 ± 0.028&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>FD</td>
<td>0.218 ± 0.054&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.259 ± 0.054&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.306 ± 0.038&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.286&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.307&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>MS</td>
<td>0.217 ± 0.052&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.308 ± 0.045&lt;sup&gt;4&lt;/sup&gt;</td>
<td>[0.308]&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.326 ± 0.036&lt;sup&gt;4&lt;/sup&gt;</td>
<td>[0.307]&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>MD</td>
<td>0.236 ± 0.051&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.346 ± 0.048&lt;sup&gt;4&lt;/sup&gt;</td>
<td>[0.306]&lt;sup&gt;4&lt;/sup&gt;</td>
<td>[0.326]&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>SS</td>
<td>0.174 ± 0.091</td>
<td>0.120 ± 0.086</td>
<td>0.120 ± 0.086</td>
<td>0.244 ± 0.051&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.243 ± 0.051&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>0.245 ± 0.094&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.347 ± 0.073&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.347 ± 0.073&lt;sup&gt;4&lt;/sup&gt;</td>
<td>[0.244]&lt;sup&gt;4&lt;/sup&gt;</td>
<td>[0.243]&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>DD</td>
<td>0.261 ± 0.088&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.235 ± 0.088&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.235 ± 0.088&lt;sup&gt;4&lt;/sup&gt;</td>
<td>[0.244]&lt;sup&gt;4&lt;/sup&gt;</td>
<td>[0.243]&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>≤0.001</td>
<td>General model 1</td>
<td>General model 2</td>
<td>General model 2</td>
<td>General model 2</td>
</tr>
<tr>
<td>$\chi^2$ (df)</td>
<td>4611.47</td>
<td>4098.27</td>
<td>4099.10</td>
<td>4101.07</td>
<td>4101.46</td>
</tr>
</tbody>
</table>

Alternate model
- General model 1
- General model 2
- General model 2

$P$-values
- $P$ ≤ 0.001 (compared with zero).
- $P$ ≤ 0.01 (compared with zero).
- $P$ ≤ 0.05 (compared with zero).
- $P$ ≤ 0.01 (compared with zero).

<sup>1</sup> General models estimated all 8 correlations: GM1 used crude values and GM2 used adjusted values for age, oral contraceptive use, vitamin E intake (diet and supplements), and cholesterol, triacylglycerol, and apolipoprotein A-I concentrations. Submodels used adjusted values for the above-listed covariates: SM1 (no sex effect on parents correlations: FS = FS and MD = MD), SM2 (no sex effect on offspring correlations: FS = FS, MD = MD and SS = SS = DD), and SM3 (no sex effect on parents or offspring correlations: FS = FS = MD = MD and SS = SS = DD). Values in brackets were constrained to be equal to a preceding value according to the hypotheses of the submodel. FM, father-model pair; FS, father-son pair; FD, father-daughter pair; MS, mother-son pair; MD, mother-daughter pair; SS, son-son pair; SD, son-daughter pair; DD, daughter-daughter pair.

<sup>2</sup> Correlation coefficient ± SE (all such values).

<sup>3</sup> $P$ ≤ 0.01 (compared with zero).

<sup>4</sup> $P$ ≤ 0.05 (compared with zero).

<sup>5</sup> Logarithm of likelihood function.

TABLE 7
Variance components of serum retinol concentrations<sup>4</sup>

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
<th>Model 5</th>
<th>Model 6</th>
<th>Model 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$(\sigma_G^2 + \sigma_H^2 + \sigma_I^2)$</td>
<td>$(\sigma_G^2 + \sigma_H^2 + \sigma_I^2)$</td>
<td>$(\sigma_G^2 + \sigma_H^2 + \sigma_I^2)$</td>
<td>$(\sigma_G^2 + \sigma_H^2 + \sigma_I^2)$</td>
<td>$(\sigma_G^2 + \sigma_H^2 + \sigma_I^2)$</td>
<td>$(\sigma_G^2 + \sigma_H^2 + \sigma_I^2)$</td>
<td>$(\sigma_G^2 + \sigma_H^2 + \sigma_I^2)$</td>
</tr>
<tr>
<td>Polygenic variance</td>
<td>0.305</td>
<td>0.517</td>
<td>[0]</td>
<td>[0]</td>
<td>0.306</td>
<td>0.259</td>
<td>0.261</td>
</tr>
<tr>
<td></td>
<td>[0.305]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0.517]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0.306]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0.259]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0.261]&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Household variance</td>
<td>0.142</td>
<td>0.272</td>
<td>[0]</td>
<td>[0]</td>
<td>0.142</td>
<td>0.090</td>
<td>0.087</td>
</tr>
<tr>
<td></td>
<td>[0.142]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0.272]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0.142]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0.090]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0.087]&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Residual variance</td>
<td>0.548</td>
<td>0.467</td>
<td>0.725</td>
<td>0.997</td>
<td>0.547</td>
<td>0.568</td>
<td>0.568</td>
</tr>
<tr>
<td></td>
<td>[0.548]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0.467]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0.725]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0.997]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0.547]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0.568]&lt;sup&gt;2&lt;/sup&gt;</td>
<td>[0.568]&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>$\chi^2$ (df)</td>
<td>670.64</td>
<td>674.23</td>
<td>676.22</td>
<td>741.49</td>
<td>670.61</td>
<td>669.62</td>
<td>669.67</td>
</tr>
<tr>
<td>$P$-values</td>
<td>≤ 0.001</td>
<td>≤ 0.001</td>
<td>≤ 0.001</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

<sup>1</sup> Values were adjusted for age, oral contraceptive use, BMI, alcohol intake, and albumin, triacylglycerol, and apolipoprotein A-I concentrations and transformed in standard score (z score). Values in brackets were fixed or constrained to be equal to a preceding value according to model hypotheses. $\sigma_G^2$, $\sigma_H^2$, $\sigma_I^2$: father, mother, son, daughter additive polygenic variances; $\sigma_{Hm}$, $\sigma_{Hs}$, $\sigma_{Hd}$: father, mother, son, daughter household common variances; $\sigma_{Ed}$, $\sigma_{Em}$, $\sigma_{Ed}$: father, mother, son, daughter shared lifestyle variances.

<sup>2</sup> Logarithm of likelihood function.
candid lipid-related polymorphisms were correlated with serum retinol and α-tocopherol concentrations.

After the covariates were accounted for, our results documented the importance of the genetic effect: 30.5% and 22.1% of the total phenotypic variability for retinol and α-tocopherol, respectively. In addition, for both vitamins, household effects were significantly different from zero: 14.2% for retinol and 18.7% for α-tocopherol concentrations. The lower household effect is in accordance with the fact that the amount of retinol in the circulating body pool is highly regulated and is essentially homeostatically controlled when liver stores are adequate (34) in populations in which vitamin A status was optimum. Except for supplement use, serum retinol concentrations were not altered when values were adjusted, especially for diet intake, and variance components did not vary significantly when families shared meals at home.

Because our study was done on a random subsample of 383 families of the overall Stanislas population, conclusions from this subsample should be valid for families living in the east of France with similar characteristics. Comparisons of our results with those of studies to come should take into account the characteristics of these French nuclear families. In fact, estimates of effects attributable to genes and to shared households depend on numerous factors: study design, type of related individuals included in the sample population (nuclear families or twins), inclusion of subjects with extreme values, distribution of environmental factors (eg, the range of vitamin intakes from foods or supplements), the covariates included in the model, and expression of variance components (proportion of the total variance or of residual variance after adjustment for covariates). In addition, variance component analysis requires strict assumptions, such as additive effects of multiple genes, environment factors, family environment, and cultural transmission (33). The assumptions of these models may have a marked effect on their results, particularly the tendency to overestimate heritability because of 3 main issues: 1) genotypic variance may include shared environmental variance that has not been removed by design or analysis, 2) estimates could greatly differ across populations according to the distribution of environmental and genetic factors, and 3) the assumption of independence between genotype and environment is likely to be violated when covariation and interaction are present.

The heritability estimate for serum retinol concentrations was higher than the shared household component: 30.5% compared with 14.2%, respectively. The lower household effect is in accordance with the fact that the amount of retinol in the circulating body pool is highly regulated and is essentially homeostatically controlled when liver stores are adequate (34) in populations in which vitamin A status was optimum. Except for supplement use, serum retinol concentrations were not altered when values were adjusted, especially for diet intake, and variance components did not vary significantly when families shared more meals at home. Heritability (additive genetic factors) could be due to numerous systems, proteins, and enzymes that are involved in retinol metabolism: 1) biliary acid synthesis, micelle formation, and solubilization of ingested fat, sterols, and fat-soluble vitamins (37, 38); 2) cleavage of provitamin A carotenoid to retinal by the cytosolic enzyme

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**TABLE 8**

Variance components of serum α-tocopherol concentrations

<table>
<thead>
<tr>
<th>Model</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
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<td>( \sigma^2_{E1} )</td>
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<td>( \sigma^2_{H1} )</td>
<td>( \sigma^2_{H2} )</td>
<td>( \sigma^2_{G1} + \sigma^2_{H1} )</td>
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<td>Residual variance</td>
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<td>0.722</td>
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<td>0.587</td>
<td>0.579</td>
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<td>Model 1</td>
<td>Model 1</td>
<td>Model 1</td>
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<td>14.08 (1)</td>
<td>5.8 (1)</td>
<td>138.58 (2)</td>
<td>0.30 (3)</td>
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<td>—</td>
<td>( \leq 0.001 )</td>
<td>( \leq 0.05 )</td>
<td>( \leq 0.001 )</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

1 Values were adjusted for age, oral contraceptive use, vitamin E supplementation and diet intake, and cholesterol, triacylglycerol, and apolipoprotein A-I concentrations and transformed in standard score (z score). Values in brackets were fixed or constrained to be equal to a preceding value according to model hypotheses.

2 Logarithm of likelihood function.
β-carotene-15,15'-dioxygenase (39); 3) retinol and retinal binding by cellular RBP type 2 and conversion of retinol into retinol by retinal reductase (40); 4) reesterification of retinol by lecithin: retinol acyltransferase (41, 42); and 5) carrying of retinol from its storage site in the liver to peripheral target tissues involving the RBP-TTR complex (8, 9, 43, 44). Concerning RBP, such major genetic defects (eg, 2 mutations in different exons) induce a severe biochemical vitamin A deficiency with low concentrations of plasma retinol and RBP, but a single mutation does not result in pathological symptoms (8).

Contrary to serum retinol concentrations, heritability and current household effects on serum α-tocopherol concentrations were of similar magnitude: 22.1% and 18.7%, respectively. Moreover, family resemblance of serum α-tocopherol concentration was not altered when covariates were taken into account and household component of variance was not improved when families shared more meals at home. In agreement, correlations between dietary and plasma or serum α-tocopherol concentrations were low and consistent with those found in the literature (45, 46). Low correlations may be due, at least in part, to the relatively narrow range of vitamin E intake from foods found in this study, and genetic differences in absorption and metabolism may contribute to the poor correlation between dietary and plasma α-tocopherol. The contribution of current household may result from shared household behavior not taken into account in our analysis. Although significant, the heritability of serum α-tocopherol concentrations was weaker than for serum retinol and may have been due to numerous systems. The first candidate is α-tocopherol transfer protein (α-TTP), a liver protein implicated in the selective retention of α-tocopherol from dietary vitamin E and for its transfer into nascent VLDL. This key phase is the major determinant of plasma α-tocopherol concentrations in humans (12). Mutations in the α-TTP gene have been detected in patients with low serum α-tocopherol concentrations and ataxia with isolated vitamin E deficiency (11, 47–50). In addition, any abnormality in chylomicron and VLDL synthesis could alter the transport of fat-soluble vitamins, eg, a defect of microsomal triacylglycerol transfer protein (51–53).

In summary, our investigation showed for the first time that serum retinol and α-tocopherol concentrations are under genetic control in healthy French families. Future studies are warranted to identify the key genetic variants that regulate the serum concentrations of these 2 vitamins in the physiologic state. Polymorphisms of genes related to proteins and enzymes involved in absorption, transport, and mobilization of retinol and α-tocopherol could contribute to similarities in vitamin concentrations within families.

We are deeply grateful for the cooperation of the families participating in the Stanislas Family Study. We acknowledge the management, reception, preclinical, laboratory, and medical staff of the Center for Preventive Medicine of Vandoeuvre-lès-Nancy (France). We especially thank Sylvie Péchéné for the collection of food intake data; Maryvonne Chauvassart and Chantal Lafaurie for family recruitment; Sylvie Michel, Véronique Michaud, Line Grandcolas, and Dominique Aguilhon for technical assistance with the biochemical assays; and Edith Leconte for coordinating the field work. We also thank David Tréguet (INSERM U 525, Paris) for providing the program package of familial correlation computation.

SG and BH designed this specific study, performed the statistical analysis, and wrote the manuscript. PL was responsible for the laboratory analyses and helped write the manuscript. RG contributed to the statistical analysis, the interpretation of the data, and the writing of the manuscript. GS and SV are the principal investigators of the Stanislas Family Study and helped write the manuscript. None of the authors had any financial or personal conflict of interest.

REFERENCES

Low dietary zinc decreases erythrocyte carbonic anhydrase activities and impairs cardiorespiratory function in men during exercise\(^1\text{–}^4\)

Henry C. Lukaski

**ABSTRACT**

**Background:** The role of zinc in promoting physiologic function during exercise is not well understood. Although some zinc-containing enzymes are postulated to regulate energy expenditure, data are limited on the effect of restricted dietary zinc on metabolic responses during exercise.

**Objective:** This study determined the effects of low zinc intake on carbonic anhydrase activity in red blood cells (RBCs) and cardiorespiratory function during exercise.

**Design:** In this double-blind, randomized crossover study, 14 men aged 20–31 y were fed low-zinc and supplemented (3.8 and 18.7 mg/d) diets made up of Western foods for 9-wk periods with a 6-wk washout. Peak work capacity, determined by using a cycle ergometer and a graded, progressive protocol, and a prolonged submaximal test (70% peak intensity for 45 min) were administered during the second and ninth weeks of each diet period.

**Results:** Dietary zinc did not affect hemoglobin or hematocrit. Low dietary zinc resulted in lower \((P < 0.05)\) serum and erythrocyte zinc concentrations, zinc retention, and total carbonic anhydrase and isoform activities in RBCs. Peak oxygen uptake, carbon dioxide output, and respiratory exchange ratio were lower \((P < 0.05)\), and ventilatory equivalents for metabolic responses during exercise were greater \((P < 0.05)\), with low than with supplemental zinc intake. Similar functional responses were observed during prolonged, submaximal exercise.

**Conclusion:** These findings indicate that low dietary zinc is associated with significant reductions in zinc status, including RBC carbonic anhydrase activities, and impaired metabolic responses during exercise. Am J Clin Nutr 2005;81:1045–51.

**KEY WORDS** Zinc depletion, erythrocyte carbonic anhydrase, cardiorespiratory function, humans

**INTRODUCTION**

Public health organizations encourage increased physical activity and consumption of healthful diets as preventive measures to reduce obesity and other chronic diseases among children and adults \((1, 2)\). These recommendations underscore concerns about the interactions between nutrient intakes and physiologic function during exercise. Although considerable information exists about the roles of macronutrients in facilitation of adaptation to physical activity and promotion of health \((3)\), there is a paucity of research data on which to develop recommendations for intakes of minerals, except iron \((4)\), in the development of physical fitness and health. One mineral that is attracting interest among the public is zinc.

Epidemiologic studies show that many adults and children may not consume adequate dietary zinc. Estimates of the proportion of adults aged 19–50 y with inadequate zinc intakes range from \(\approx 20\%\) in men to nearly \(40\%\) in women \((5)\). Surveys of physically active persons also indicate that low dietary zinc is common, particularly among individuals who participate in aerobic activities \((6, 7)\), such as those recommended to promote health and well-being \((1, 2)\).

Low zinc status hampers the physiologic functions required for optimal work performance. Low zinc intakes and reduced serum zinc concentrations have been associated with impaired muscle function, including reduced strength and increased propensity to fatigue \((8, 9)\), and decreased power output during peak work capacity testing \((10)\). Thus, low zinc status may lead to reduced physical function and performance.

Because zinc-containing enzymes number \(>200\) in mammalian systems \((11, 12)\), any effect of dietary zinc may be translated into metabolic and functional defects by zinc metalloenzymes. Carbonic anhydrase \((EC\ 4.2.1.1)\), a zinc metalloenzyme, catalyzes the reversible hydration and dehydration of carbon dioxide, a product of cellular aerobic energy production \((12)\). Removal of zinc from this metalloenzyme inactivates the enzyme \((13)\). Studies in various species, including rodents, domestic fowl, calves, and lambs, have found that dietary zinc deficiency significantly reduces red blood cell (RBC) carbonic anhydrase activity \((14–19)\) and, in a few cases, impairs respiratory function \((14, 15)\). Sickle-cell anemia patients with biochemically determined zinc and other nutritional deficiencies had significantly decreased carbonic anhydrase protein in RBCs that increased significantly...
with zinc supplementation (20). Thus, reduced carbonic anhydrase activity in RBCs may be an indicator of zinc deficiency, particularly in humans.

The present study tested the hypothesis that restricted, compared with increased, dietary zinc in amounts consumed by physically active men would decrease zinc status and retention and RBC carbonic anhydrase activity and impair physiologic responses during controlled exercise.

SUBJECTS AND METHODS

Experimental design

Men engaged in regular physical activities, recreational or employment related, participated in a double-blind, crossover feeding study. On admission to the study, the men were matched by body mass index and assigned to receive either a basal low-zinc diet [3.5 mg/10.5 MJ (2500 kcal)] or the basal diet supplemented with 15 mg Zn as zinc sulfate (ZnSO₄ · 7H₂O) added to juice. These amounts of dietary zinc are consistent with reported zinc intakes of young men involved in various regular physical activities but not intense physical training (7). The volunteers consumed each diet for 9 wk, followed by a 6-wk washout period, and then received the other diet for 9 wk. The men lived in their usual residences and maintained their usual lifestyles with the exception of consuming the food and beverages provided and participating in scheduled testing. Phlebotomy, collection of excreta for determinations of zinc nutritional status and balance, and physiologic testing were performed at specified times during each dietary period.

Subjects

Fourteen men aged (± SEM) 28.6 ± 1.3 y (range: 20–36 y) with a mean body weight of 86.2 ± 3.4 kg (range: 70–110 kg), a body mass index (in kg/m²) of 24.9 ± 0.8 (range: 21.9–28.7), and fat-free mass of 66.9 ± 1.5 kg (QDR 2000W; Hologic Inc, Waltham, MA) completed all aspects of the study. The men were recruited through public advertisements and were selected after medical, nutritional, and psychological evaluations established that they had no underlying disease and were emotionally suited to participate in the clinical research project. The men gave their written informed consent before participation in this study, which was approved for human subjects by the Institutional Review Board of the University of North Dakota and by the Human Studies Review Committee of the US Department of Agriculture.

Diets

Registered dietitians planned the basal low-zinc diet that was composed of Western foods presented in a 3-d rotating menu cycle (Table 1). The energy distribution of the diet was 12% protein, 30% fat, and 58% carbohydrate. The basal, low-zinc diet was calculated (21) to supply recommended amounts of all essential nutrients except zinc. Limited amounts of salt, pepper, and selected low-energy carbonated beverages were individualized to the preference of each volunteer and then served consistently throughout the study. The range of daily energy intakes was 7.5–16.0 MJ (1750–3750 kcal).

All diet ingredients, except water, were weighed, prepared, and provided to the volunteers by the dietary staff. Volunteers ate
one meal at the US Department of Agriculture Agricultural Research Service Grand Forks Human Nutrition Research Center on weekdays and consumed the remaining foods, after minimal reheating, away from the center. Foods were weighed to 0.1 g accuracy and were consumed quantitatively. The volunteers agreed to consume only the food and beverages provided by the dietary staff. On the basis of self-reports, the men did not consume other food or beverages.

**Chemical analyses**

**Blood collection**

Fasting venous blood samples were taken by phlebotomy during the second and ninth week of each dietary period and were limited to 50 mL/mo for routine health assessment and determination of zinc status. Blood was drawn into plastic syringes from an antecubital vein, which had been made visible by temporary use of a tourniquet, after the volunteers had fasted for 10 h. Samples were collected and prepared with care to avoid hemolysis. Hemoglobin and hematocrit were determined by using an automated clinical analyzer (Cell Dyn-3500; Abbott Instruments, Chicago, IL). Serum was processed within 90 min of the time the blood was obtained.

**Zinc in serum and red blood cells**

Serum for zinc determination was diluted 1:5 with distilled-deionized water; serum zinc was measured by using flame atomic absorption spectrometry with standards in a 5% glycerol matrix (22). Aliquots of hemolyzed RBCs were diluted and deproteinized with trichloroacetic acid and were then analyzed for zinc by using atomic absorption spectrometry (23). Concurrent analysis of SeraChem I controls (Fisher Scientific, Orangeberg, NY) yielded values of 146.8 ± 6.0 (x ± SD; n = 60) μmol/L (96 ± 9 μg/dL) as compared with certified values of 140.2 ± 1.4 μmol/L (92 ± 9 μg/dL) for zinc concentration.

**Carbonic anhydrase activities**

Another venous blood sample was collected in a syringe containing 20 U heparin/mL; this amount of heparin, which contains negligible zinc, has been shown to not be a source of contamination in determinations of zinc in plasma and RBCs (24). After centrifugation (2800 × g at 4 °C for 15 min), plasma and white blood cells were removed, and packed RBCs were washed 3 times with normal saline followed by centrifugation. After the last wash, the supernatant fluid was removed and the packed RBCs were lysed with an equal volume of distilled-deionized water. Samples were centrifuged to remove cellular debris.

To determine carbonic anhydrase activity, aliquots of hemolyzed RBCs were diluted 1:50 and 1:100 with distilled-deionized water (25). An experienced analyst bubbled carbon dioxide through 5 mL cold distilled-deionized water (either with or without sample) for 1 min. Two milliliters of 50-mmol/L, barbital buffer (pH = 7.9) was added, and the time to attain a pH of 6.7 was recorded. A calibrated pH meter (Advanced pH Meter, model #840035; Technika, Phoenix, AZ) and fast-reacting electrode (#840016; Technika) were used to monitor pH in the reaction mixture (26). The 1:100 diluted sample gave the total carbonic anhydrase activity. The procedure was repeated with the 1:50 diluted sample, which was diluted again 1:1 with a solution containing 40 mmol bromopyruvate/L and 47 mmol Na2HPO4/L (pH = 7.5). The pH change was monitored as described above, which yields an estimate of carbonic anhydrase II activity (25, 26). Carbonic anhydrase isozyme I activity was estimated as the difference between total and isozyme II activities. The hemoglobin concentration of each hemolysate (mg hemoglobin/mL hemolysate) was determined with an automated cell counter. One enzyme unit is defined as the quantity of sample needed to result in a doubling of the reaction time without sample (26). Variability in carbonic anhydrase activity determinations was 2–3% within and 4–5% between days.

**Zinc in food and excreta**

All food was weighed with an accuracy of 0.1 g during preparation in the metabolic kitchen. Urine and feces were collected carefully to avoid trace mineral contamination. Duplicate diets at the 10.6-MJ (2500-kcal) intake were prepared daily for analysis and were blended in a plastic blender with stainless steel blades. Adjustments for differences in individual energy intakes were calculated proportionately.

The zinc content of 4 consecutive 3-d composites of diets and feces, obtained during the last 12 d of each dietary period, was determined by inductively coupled argon-plasma emission spectrophotometry (ICAP; Jarrell-Ash, Waltham, MA) after wet digestion of aliquots of freeze-dried, blended material with nitric and perchloric acids (23, 27). Urinary zinc, collected during the same 12-d periods, was determined by ICAP analysis of a diluted aliquot (23).

Concurrent replicate analysis of zinc in bovine liver samples (standard reference material 1577b; US National Institute of Standards and Technology, Gaithersburg, MD) yielded a value of 1.807 ± 0.037 (x ± SD; n = 14) μmol/g (118.1 ± 2.4 μg/g) for zinc concentration as compared with a certified value of 1.882 ± 0.122 μg/g. Duplicate analysis of a fecal pool sample (n = 14) yielded a value of 8.32 ± 0.02 μmol/g.

Zinc balance was calculated as the difference between intake and excretion (feces plus urine) during the last 12 d of each dietary period (ie, 4 consecutive 3-d menu rotation periods).

**Metabolic responses at rest and during exercise**

Two types of exercise tests were administered: a graded, progressive, peak performance test and a prolonged submaximal test. Peak work capacity was determined by using a cycle ergometer (Monark; Varberg, Sweden) and a continuous, progressive exercise protocol that was terminated when the volunteer reached voluntary exhaustion. The submaximal test included a 5-min warmup at a work load equal to 50% peak oxygen uptake followed by 45 min at a work load of 70% peak oxygen consumption.

Exercise tests were performed during the second and ninth week of each dietary period. Two work capacity tests were performed on nonconsecutive days at each designated time period. The first test was used to acclimate or reacquaint the volunteer with the test environment and equipment; data from the second test were used to determine dietary effects on metabolic responses. Only one prolonged submaximal test was administered at the second and ninth week of each dietary treatment; it occurred within 7 d of completion of the second peak work capacity test. After an overnight fast, each volunteer rested for 5 min while seated on the ergocycle and then pedaled at 70 revolutions/min starting with an initial resistance of 0 kilopond (kP) and increasing by 0.5 kP every 3 min until he could not maintain the pedaling
TABLE 2
Zinc intake, excretion, and retention determined in 14 men¹

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<thead>
<tr>
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<th>Zinc-supplemented diet</th>
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<td>Intake</td>
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<td>3.68 ± 0.02</td>
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<td>Urine</td>
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<td>Feces</td>
<td>16.78</td>
<td>3.08</td>
<td>&lt;0.05</td>
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<tr>
<td>Retention</td>
<td>1.46</td>
<td>0.23</td>
<td>&lt;0.05</td>
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</table>

¹ 12-d period (4 consecutive 3-d menu rotations).
² ± SEM (all such values).

Results of repeated-measures analysis of variance (ANOVA) with individual volunteers used as their own controls (28) were used to determine dietary treatment and time effects, with Tukey-Kramer contrasts, and to determine whether the order of presentation of the diets had an effect. There were no order effects for any dependent variables. Variance in the data is expressed as a pooled SD, which was calculated as the square root of the mean square error from the ANOVA. Because diet analyses used fewer independent samples (n = 5), only means and SEMs are presented for these data. Tests were done to determine whether zinc balance data were different from 0 (28).

RESULTS

Body weight was maintained within ±2% of admission weight, and body composition was unchanged during the experiment (data not shown).

Zinc intake, excretion, and balance

Zinc excretion corresponded with zinc intake (Table 2). Urinary and fecal zinc losses were significantly lower with dietary zinc restriction. Zinc balance was significantly lower with the low than with the supplemental zinc intake. Zinc balance (intake − excretion) was significantly different (P < 0.05) from 0 during consumption of the zinc-supplemented diet; zinc balance was not significantly different from 0 during the low-zinc diet.

Blood biochemical measures and indexes of zinc status

Zinc intake affected neither hemoglobin concentration nor total RBC count (Table 3). Serum zinc and RBC zinc concentrations, total carbonic anhydrase activity, and the activities of the carbonic anhydrase I and II isoforms in RBCs decreased significantly over time when the men consumed the low-zinc diet but did not change significantly when they ate the supplemental-zinc diet. Total carbonic anhydrase activity was significantly correlated (r = 0.92, P < 0.01) with erythrocyte zinc concentration.

Functional responses during exercise

Peak oxygen uptake values ranged from 2.9 to 4.1 L/min or 43–50 mL·kg⁻¹·min⁻¹ determined with high dietary zinc. Zinc intake affected exercise performance as well as functional responses during graded, peak exercise. The duration of exercise time, and hence the total work, to attain peak work performance tended to be less (≈6%) when dietary zinc was low (Table 4). Peak ventilatory volume and ventilatory equivalents for oxygen and carbon dioxide increased significantly, and peak oxygen consumption, carbon dioxide output, and respiratory exchange ratio decreased significantly over time when the volunteers received the low-zinc diet with no change when the men consumed the supplemental-zinc diet.

TABLE 3
Hematology and blood biochemical indicators of zinc status of 14 men consuming diets with 2 zinc intakes⁴

<table>
<thead>
<tr>
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<td>2 wk 9 wk</td>
<td>Pooled SD</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
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<td>147 148</td>
<td>1.1</td>
</tr>
<tr>
<td>RBC (1 × 10³/mL)</td>
<td>5.0 5.0</td>
<td>5.0 5.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Serum zinc (µmol/L)</td>
<td>13.9 13.9</td>
<td>13.8 10.9</td>
<td>0.4</td>
</tr>
<tr>
<td>RBC zinc (nmol/g Hb)</td>
<td>33.1 33.1</td>
<td>33.0 25.6</td>
<td>2.8</td>
</tr>
<tr>
<td>Total CA activity (U/g Hb)</td>
<td>1894 1986</td>
<td>1946 1478</td>
<td>103</td>
</tr>
<tr>
<td>CA I activity (U/g Hb)</td>
<td>691 689</td>
<td>678 542</td>
<td>48</td>
</tr>
<tr>
<td>CA II activity (U/g Hb)</td>
<td>1281 1298</td>
<td>1251 938</td>
<td>65</td>
</tr>
</tbody>
</table>

⁴ RBC, red blood cell; Hb, hemoglobin; CA, carbonic anhydrase; CA I and CA II, isoforms of carbonic anhydrase. Values in the same row with different superscript letters are significantly different, P < 0.05 (Tukey-Kramer contrast).
Functional and metabolic responses to peak exercise of 14 men consuming diets with 2 zinc intakes

Low zinc intake tended to impair performance and alter physiologic function during the submaximal exercise test. When dietary zinc was low, 4 of the volunteers did not complete the 45-min test, which resulted in a decrease (11%; $P = 0.10$) in the average duration of submaximal exercise (Table 5) and total work performed. Heart rate, ventilatory volume, and ventilatory equivalents for oxygen and carbon dioxide were significantly increased, whereas oxygen consumption, carbon dioxide output, and respiratory exchange ratio were significantly decreased, over the time the men consumed the low-zinc diet but did not change significantly when they ate the supplemental-zinc diet.

### DISCUSSION

The consequences of restricted dietary zinc depend on the physiologic state of the individual, the amount and bioavailability of the ingested zinc, and the duration of the low zinc intake (29). The present findings that low, compared with supplemental, dietary zinc result in significantly lower zinc retention, serum and RBC zinc concentrations, and RBC carbonic anhydrase activities indicate marginal zinc deficiency. Although zinc balance was similar to 0 (0.23 mg/d; 30) at a dietary zinc level of 3.8 mg/d suggests net zinc loss. Similarly, use of 0.31 mg of surface zinc loss for a zinc intake of 18.8 mg/d (30) reduces zinc balance from 1.46 to 1.15 mg/d. Importantly, zinc loss was associated with significantly lower RBC carbonic anhydrase activities with low compared with supplemental dietary zinc.

Previous studies found decreased RBC total carbonic anhydrase activity in animals and fowl fed low-zinc diets. Early studies of zinc-deficient rats (14) and chicks (15) reported gasping respiration and increased rates of respiration with significantly decreased RBC total carbonic anhydrase activity. Zinc-deficient calves (16), lambs (17), and rats (18, 19) also had decreased RBC carbonic anhydrase activities. The reduction of RBC carbonic anhydrase activity depended on the amount and duration of zinc restriction; it ranged from 35% to 60% of that in controls fed zinc-adequate diets (31).

Reports of the responsiveness of RBC carbonic anhydrase to zinc intake in humans are limited. Prasad et al (20) studied patients with sickle cell anemia and found zinc deficiency and significantly lower plasma and RBC zinc concentrations than in healthy adults. The RBC carbonic anhydrase isoforos I and II proteins were 40% less than values in zinc-adequate controls; after oral zinc therapy (150 mg/d for 4–60 wk), isozyme proteins increased by >20% in the zinc-deficient patients. Zinc supplementation significantly increased RBC and plasma zinc. Prasad

### TABLE 5

Physiologic responses during prolonged, submaximal exercise of 14 men consuming diets with 2 zinc intakes

<table>
<thead>
<tr>
<th></th>
<th>Zinc-supplemented diet</th>
<th>Low-zinc diet</th>
<th>$P$ (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 wk</td>
<td>9 wk</td>
<td>2 wk</td>
</tr>
<tr>
<td>Duration (min)</td>
<td>45.5</td>
<td>45.0</td>
<td>45.1</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>144$^a$</td>
<td>145$^a$</td>
<td>146$^b$</td>
</tr>
<tr>
<td>$V_\text{E}$ (L/min)</td>
<td>70$^a$</td>
<td>71$^a$</td>
<td>70$^a$</td>
</tr>
<tr>
<td>$\text{VO}_2$ (L/min)</td>
<td>2.39$^a$</td>
<td>2.41$^a$</td>
<td>2.42$^b$</td>
</tr>
<tr>
<td>$\text{VCO}_2$ (L/min)</td>
<td>2.34$^a$</td>
<td>2.39$^a$</td>
<td>2.37$^b$</td>
</tr>
<tr>
<td>RER</td>
<td>0.98$^a$</td>
<td>0.99$^a$</td>
<td>0.98$^b$</td>
</tr>
<tr>
<td>$V_\text{E}/\text{VO}_2$</td>
<td>29.2$^a$</td>
<td>29.2$^a$</td>
<td>28.9$^b$</td>
</tr>
<tr>
<td>$V_\text{E}/\text{VCO}_2$</td>
<td>29.9$^a$</td>
<td>29.7$^a$</td>
<td>29.5$^a$</td>
</tr>
</tbody>
</table>

1 HR, heart rate; $V_\text{E}$, ventilatory volume; $\text{VO}_2$, oxygen uptake; $\text{VCO}_2$, carbon dioxide exhaled; RER, respiratory exchange ratio; $V_\text{E}/\text{VO}_2$, ventilatory equivalent for oxygen; $V_\text{E}/\text{VCO}_2$, ventilatory equivalent for carbon dioxide. Values in the same row with different superscript letters are significantly different, $P < 0.05$ (Tukey-Kramer contrast).
et al (20) concluded that synthesis of the carbonic anhydrase apoenzyme requires zinc and that the apoenzyme does not accumulate if zinc is lacking. Canfield and Johnson (32) reported a significant correlation ($r = 0.89$) between RBC total carbonic anhydrase activity and dietary zinc ranging from 3 to 17 mg/d in men. Men fed graded amounts of dietary zinc (1, 2, 3, 4, and 10 mg/d) for 36-d periods had no changes in plasma zinc or RBC carbonic anhydrase activity (33). The brief duration of the treatment periods was probably insufficient to affect zinc status; no effects of dietary zinc were found on the activities of other zinc metalloenzymes.

Use of carbonic anhydrase activity as a putative mechanism linking low zinc status with physiologic functions supporting physical activity has a strong theoretical basis. Carbonic anhydrase was the first zinc-containing enzyme described (13), and isoforms are present in all organisms. At least 9 different isozymes are distributed in mammalian tissues, including RBCs, kidney, muscle, bone, and other organs (34). This broad distribution reflects varying capacities for carbon dioxide removal and acid-base regulation. Three isoforms of carbonic anhydrase are found in RBCs. Isoform I, which is present in the greatest concentration, has limited carbon dioxide hydration-dehydration capacity. Isoform II is the physiologically most important for carbon dioxide removal but is moderate in concentration. The third isoform has a negligible role in carbon dioxide transport.

Carbonic anhydrase is also found in skeletal muscle. Red oxidative muscle contains 3–5 times more zinc than does white glycolytic muscle (35); the zinc is found in carbonic anhydrase (36). Skeletal muscle contains 2 isoforms of carbonic anhydrase, III and IV (37). Isoform III protects against free radical damage and controls the intermediary metabolism of glucose and fat, whereas isoform IV facilitates carbon dioxide removal (38). Thus, the ubiquitous distribution of carbonic anhydrases in mammalian tissues and their heterogeneous roles in cellular energy metabolism provide a novel link between zinc and energy expenditure.

A low zinc intake, which is characteristic of certain groups of the US population [including adolescents, the elderly, and some physically active individuals (5, 7)], consumed for 9 wk resulted in significantly lower total and specific carbonic anhydrase isozyme activities in RBCs than did a diet supplemented with zinc. The decreased enzyme activity was found in parallel with impaired cardiorespiratory responses during intense and prolonged submaximal exercise. The blunted oxygen uptake and carbon dioxide elimination and decreased respiratory exchange ratio are consistent with the findings of Wada and King (39), who showed decreased resting energy expenditure and a reduced respiratory exchange ratio in young men fed 5.5 compared with 16.5 mg Zn. Thus, suboptimal zinc intake is associated with disturbances in energy metabolism and cardiorespiratory function.

The effect of low zinc on carbonic anhydrase activity may be useful in explaining previous reports of altered muscle function and exercise capacity in humans with low zinc status. Studies of adolescents and men with significant reductions in serum zinc concentrations and decreased muscle strength and work capacity may be explained by reduced carbonic anhydrase activity (9, 10). Similarly, men fed diets low in zinc responded with significant decreases in plasma zinc and reduced muscular strength (8). Conversely, increased strength gain and muscle endurance of older women supplemented with zinc (30 mg/d) may be the result of increased activity of tissue carbonic anhydrase (40). Collectively, these findings support a role for zinc, as related to carbonic anhydrase, in the promotion and maintenance of physical activity and performance and merit further research.

A previous study of young men fed a diet low in zinc (=4 mg/d) and then supplemented with zinc (35 mg/d) failed to show any effect of zinc intake on peak work capacity, metabolic responses during exercise, zinc balance, or zinc status indicators (41). In contrast, the present study found net zinc loss, decreases in biochemical markers of zinc status, and altered metabolic responses to controlled exercise. Thus, reduced zinc status is associated with impaired physiologic responses during work.

Certain technical factors limited past success of carbonic anhydrase as a marker of human zinc nutritional status. Reliance on a colorimetric method (25) contributed to a general lack of precision and increased interobserver variability (26). However, the use of highly accurate and precise automated pH devices and electrodes has eliminated reproducibility as a problem (26). A second potential obstacle has been the use of short durations of dietary zinc interventions. Failure to use periods of ≥60 d, consistent with the half-life of RBCs, apparently reduced the likelihood of finding changes in carbonic anhydrase. Thus, future studies that seek to assess the sensitivity of this zinc metalloenzyme to dietary zinc might benefit from the use of cell-separation techniques that enable a comparison of carbonic anhydrase activity and apoenzyme protein expression in newly formed compared with older erythrocytes, as well as incorporation of more modern assay procedures.

In conclusion, the present study showed that low dietary zinc in amounts consumed by segments of the population regularly involved in regular physical activity reduces zinc status. Our results provide evidence in support of the use of RBC carbonic anhydrase activity as a useful marker of human zinc nutritional status and links its in vitro reduction in activity to functional impairments in respiratory and metabolic responses to different types of exercise. These findings provide the first hypothesis explaining previous observations of impaired muscle function with reduced circulating zinc concentrations in adolescents and adults and emphasize the potential of carbonic anhydrase measurement in future studies examining zinc intake and physical activity.

The author gratefully recognizes the valuable contributions of other members of our human studies research team: BS Hoverson supervised diet development and preparations, SK Gallagher supervised clinical and mineral analysis laboratories, CB Hall and WA Siders administered the exercise tests and collected indirect calorimetry data, and KG Michelsen performed the carbonic anhydrase assays. I am particularly thankful for the dedication and conscientious participation of the men, without whose contributions this study would not have been successful.

HCL conceptualized and designed the study, supervised the data collection and analyses, interpreted the findings, and wrote the manuscript. The author had no conflict of interest to report.

REFERENCES


Lower plasma α-carboxyethyl-hydroxychroman after deuterium-labeled α-tocopherol supplementation suggests decreased vitamin E metabolism in smokers

Richard S Bruno, Scott W Leonard, Jun Li, Tammy M Bray, and Maret G Traber

ABSTRACT
Background: Cigarette smoking increases the fractional disappearance rates of α-tocopherol and is associated with increased oxidative stress, but its effects on α-tocopherol metabolism are unknown.

Objective: We hypothesized that smokers would have less α-tocopherol available and consequently lower plasma α-carboxyethyl-hydroxychroman (α-CEHC), the α-tocopherol metabolite produced by a cytochrome P450–mediated process.

Design: Smokers and nonsmokers (n = 10 per group) were supplemented with deuterium-labeled α-tocopherol acetates (75 mg each d1-RRR-α-tocopheryl and d6-all-rac-α-tocopheryl acetate) from day −6 to day −1, and plasma tocopherols and CEHCs were measured (day −6 through day 17).

Results: After 6 d of supplementation, plasma d1- and d6-α-tocopherol concentrations did not differ significantly between groups. Plasma d1- and d6-α-CEHCs were detectable only from day −5 to day 5. Before supplementation, unlabeled α- and γ-CEHCs were ≈60% and 40% lower, respectively, in smokers than in nonsmokers (P ≤ 0.05). In addition, d0-, d1-, and d6-α-CEHC areas under the curves were ≈50% lower in smokers (P < 0.05), and smokers had lower maximal d1-, d6-, and d6-α-CEHC areas (P = 0.0006) concentrations. Notably, 2.9–4.7 times as much α-CEHC was produced from all-rac-α-tocopherol than from RRR-α-tocopherol. During supplementation, smokers had about one-half (P < 0.05) the plasma total, d6-, or d6-α-CEHC concentrations that nonsmokers did given similar α-tocopherol concentrations.

Conclusions: Smoking did not increase α-tocopherol disappearance through P450-mediated tocopherol metabolism. Therefore, the mechanism of increased α-tocopherol disappearance in smokers likely operates through oxidation pathways, which is consistent with α-tocopherol’s antioxidant function. Consequently, evaluating the molecular mechanism or mechanisms responsible for tocopherol metabolism under conditions of oxidative stress and the mechanisms that regulate α-tocopherol status is warranted. Am J Clin Nutr 2005;81:1052–9.

KEY WORDS Oxidative stress, carboxyethyl-hydroxychroman, CEHC, smokers, tocopherols, metabolism, cytochrome P450

INTRODUCTION
Oxidative stress, such as cigarette smoke, has been observed during in vitro investigations to deplete α-tocopherol from human plasma (1, 2). In humans, direct comparison of plasma α-tocopherol concentrations between unsupplemented smokers and nonsmokers has yielded inconsistent results: some investigations reported lower plasma α-tocopherol concentrations among smokers (3, 4), whereas other investigations observed no differences in plasma concentrations between these groups (5–7). Recently, we studied the fractional disappearance kinetics of vitamin E in smokers and nonsmokers (8). After 6 days of deuterium-labeled α-tocopherol supplementation, plasma labeled α-tocopherol concentrations were not significantly different between the groups, but fractional disappearance rates of α-tocopherol were 13% faster and α-tocopherol half-lives were 10 h shorter in smokers than in nonsmokers (8). Moreover, the fractional disappearance rates of α-tocopherol in smokers were inversely related to their plasma vitamin C concentration, which suggests that higher vitamin C status could attenuate α-tocopherol disappearance (8) and further supports the role of oxidative stress in vitamin E disappearance.

Because differences in circulating unlabeled α-tocopherol concentrations are not consistently observed between control and experimental groups, it has been suggested that additional biomarkers are necessary for the assessment of α-tocopherol status (9). Therefore, measurement of the circulating or urinary vitamin E metabolite α-carboxyethyl-hydroxychroman (α-CEHC) represents a possible means of assessing in vivo...
α-tocopherol status. CEHCs are nonoxidation products of tocopherol (Figure 1). Their formation is initiated via cytochrome P450–mediated α-oxidation, followed by stepwise β-oxidation of the phytyl tail (10–13). Subsequently, CEHCs are excreted after glucuronification or sulfation (14).

Metabolism of tocopherols and tocotrienols to their respective CEHCs plays a role in the excretion of unwanted or excess vitamin E forms (15). Urinary recovery of α- and γ-CEHCs from α- and γ-tocotrienol supplementation is only a small fraction of the administered dose (16). As for the major dietary and biological forms of vitamin E, α- and γ-tocopherol (17), γ-tocopherol appears to be more actively metabolized to γ-CEHC, as suggested by the >12 times greater plasma concentration of γ-CEHC than of α-CEHC despite significantly higher plasma concentrations of α-tocopherol relative to γ-tocopherol (18). In contrast, α-tocopherol appears to be actively metabolized only when a plasma α-tocopherol threshold of 30–40 μmol/L is reached or when individuals are supplemented (16, 19, 20). Consistent with the metabolism of excess vitamin E, the α-tocopherol transfer protein discriminates between natural (RRR) and synthetic α-tocopherols, such that 4 of the 8 stereoisomers in all-rac (2R–, not 2S-α-tocopherol forms) are maintained in the plasma (21), whereas 3 times more all-rac-α-tocopherol than RRR-α-tocopherol is metabolized to α-CEHC and excreted in the urine (22).

To date, the role of oxidative stress on vitamin E status and subsequent plasma α-CEHC concentrations has not been investigated. Therefore, our aim was to evaluate plasma deuterium-labeled α-CEHC concentrations from cigarette smokers and nonsmokers who were supplemented with deuterium-labeled α-tocopherol for 6 d (8). In the present investigation, we hypothesized that smokers, as a result of increased oxidative stress (8), would have less α-tocopherol available for α-CEHC production and consequently lower plasma α-CEHC concentrations.

FIGURE 1. Structures of unlabeled and labeled tocopherols and carboxyethyl-hydroxymethanes (CEHCs). Nonsmokers (n = 10) and smokers (n = 10) were supplemented with 75 mg each d3-RRR- and d6-all-rac-α-tocopheryl acetate on 6 consecutive evenings after a standard meal. Plasma samples obtained from day −6 to day 17 were analyzed by liquid chromatography–mass spectrometry for unlabeled (d3-α- and d6-γ-CEHC) and deuterium-labeled (d1- and d6-α-CEHC) CEHCs (vitamin E metabolites) as well as for unlabeled (d3-α- and d6-γ-tocopherol) and deuterium-labeled (d1- and d6-α-tocopherol) tocopherols.

<table>
<thead>
<tr>
<th>CEHC</th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d0-γ</td>
<td>H</td>
</tr>
<tr>
<td></td>
<td>d3-α</td>
<td>CH3</td>
</tr>
<tr>
<td></td>
<td>d3-α</td>
<td>CD3</td>
</tr>
<tr>
<td></td>
<td>d6-α</td>
<td>CD3</td>
</tr>
</tbody>
</table>

α-tocopherol status. CEHCs are nonoxidation products of tocopherol (Figure 1). Their formation is initiated via cytochrome P450–mediated α-oxidation, followed by stepwise β-oxidation of the phytyl tail (10–13). Subsequently, CEHCs are excreted after glucuronification or sulfation (14).

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SUBJECTS AND METHODS

Materials

HPLC-grade methanol and glacial acetic acid were obtained from Fisher (Fair Lawn, NJ). Trolox, phosphate-buffered saline, ascorbic acid, and β-glucuronidase (type H-1, contains minimum 300 000 U/g β-glucuronidase activity and minimum 10 000 U/g sulfatease activity) were from Sigma-Aldrich (St Louis, MO). Diethyl ether was obtained from Mallinckrodt Baker Inc (Phillipsburg, NJ). Standards, including unlabeled (d0), d3-RRR-, and d6-all-rac-α-tocopheryl acetates and d6-γ-tocopherol, were gifts from James Clark of Cognis Nutrition and Health (LaGrange, IL). all-rac-α-5,7,8-(CD3)3-tocopheryl acetate (d6-all-rac-α-tocopheryl acetate) was provided by Carolyn Good of General Mills and was synthesized by Isotec Inc (Miamisburg, OH). The isotopic purity of d6-α-tocopherol was found to be 88.4% d6 and the remainder d5, 2,5,7,8-Tetramethyl-2-(2-carboxyethyl)-6-hydroxychroman (α-CEHC) and 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (γ-CEHC, or LLU-α) were gifts from WJ Wechter of Loma Linda University (Loma Linda, CA).

Deuterated α-tocopheryl acetates

Capsules containing RRR-α-5-(CD3)3-tocopheryl acetate and all-rac-α-5,7-(CD3)3-tocopheryl acetate (d6-α-tocopheryl acetate and d6-all-rac-α-tocopheryl acetate, respectively) were a gift from the Natural Source Vitamin E Association and were synthesized by Eastman Kodak, Rochester, NY. The d3-RRR- and d6-all-rac-α-tocopheryl acetates were encapsulated in a gelatin capsule as nominal 1:1 mixtures in 150-mg quantities. The molar ratio of d6-RRR- to d6-all-rac-α-tocopherol was determined to be 0.98 (22).

Study participants

The Institutional Review Board at Oregon State University approved the protocol for this investigation, and all participants provided written consent before enrollment. An extensive description of the subjects and protocol was reported previously (8). In brief, healthy, normolipidemic volunteers (n = 10 nonsmokers and 10 smokers) were selected for this study on the basis of age (18–35 y), nonnutritional supplement use (20), and exercise status (<2 h/wk of aerobic activity). Cotinine, the metabolite of nicotine, was measured by radioimmunoassay (Diagnostic Products Corp, Los Angeles, CA) to verify smoking status. Participants were enrolled in the study only if they had clinical serum chemistry values within normal limits.

Experimental design

On 6 consecutive evenings (day −6 to day −1), the participants ingested the deuterated α-tocopheryl acetate supplement containing 75 mg each d3-RRR-α-tocopheryl acetate and d6-all-rac-α-tocopheryl acetate immediately after a standard meal (43% carbohydrate, 17% protein, and 41% fat). Blood samples were collected from the antecubital vein into tubes containing 0.05 mL 15% K3 EDTA (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) after the subjects had fasted overnight (10–12 h) on days −6, −5, −4, −3, −2, −1, 0, 1, 2, 3, 4, 5, 6, 8, 10, 13, 15, and 17 (negative days denote the supplementation period). Blood samples were kept on ice for <30 min before plasma isolation. Plasma was separated by centrifugation (500 × g, 15 min, 4 °C; Beckman TJ-6, Paola Alto, CA), aliquoted into cryovials, snap frozen in liquid nitrogen, and then stored at −80 °C until analyzed. Smokers were asked to refrain from smoking for 1 h before blood collection to alleviate transient oxidative stress effects. Last, all participants were instructed to complete a 3-d food
record (2 weekdays, 1 weekend day) during the investigation. Nutrient intakes were analyzed by using FOOD PROCESSOR dietary analysis software (version 7.9; ESHA Research Inc, Salem, OR).

Analyses

The chemical structures of labeled and unlabeled tocopherols and CEHCs are illustrated in Figure 1. Plasma labeled and unlabeled α- and γ-CEHCs were extracted (23) and measured by liquid chromatography–mass spectrometry (LC-MS) (24). The linear quantitative range for α- and γ-CEHC analysis was 0.2–20 pmol injected, and the lower limit of detection was 80 fmol injected on the column.

The following were analyzed from plasma and were previously reported (8): labeled and unlabeled tocopherols, ascorbic acid, uric acid, F2α-isoprostanes, total cholesterol, and triacylglycerols. All biochemical analyses were conducted such that all samples (day −6 to day 17) from a participant were extracted and measured in the same batch.

Statistical analyses

Statistical analysis was performed by using GraphPad PRISM (version 4.0; GraphPad Software, San Diego, CA). Total α-tocopherol or total α-CEHC refers to the sum of the corresponding unlabeled (d0-) and labeled (d3-, d6-) compounds. Area under the curve (AUC) was estimated by using the trapezoidal rule. For any undetectable CEHC values, one-half the detectable limit was substituted. An unpaired Student’s t test or two-way analysis of variance with repeated measures was used as appropriate for comparisons between smokers and nonsmokers. Simple linear regression, stratified by smoking status, was used to visualize the associations between α-tocopherol and α-CEHC and between γ-tocopherol and γ-CEHC. To further estimate the main effect and interactive effect of α-tocopherol and smoking status on α-CEHC concentrations, we conducted multiple linear regression by using SAS (SAS Institute Inc; Cary, NC) GENMOD with the generalized estimating equation to control for within-subject correlation. All data were considered statistically significant when P values were <0.05. All data are reported as means ± SEs unless otherwise noted.

RESULTS

Participant characteristics and α-tocopherol biokinetics

Complete details of deuterium-labeled α-tocopherol biokinetics were previously reported (8). Before supplementation, there were no significant differences between smokers and nonsmokers with respect to age; body mass index; plasma concentrations of ascorbic acid, uric acid, α-tocopherol, and γ-tocopherol; or dietary vitamin E intakes. Smokers had a greater degree of oxidative stress as marked by elevated F2α-isoprostanes (8).

CEHC time course and area under the curve

Plasma α- and γ-CEHC time courses for smokers and nonsmokers are shown in Figure 2 and Figure 3. As expected at baseline (before supplementation; day −6), no plasma d3-α-
CEHCs or d₆α-CEHCs were detected (Figure 2B, C). However, only 7 of 10 nonsmokers and 8 of 10 smokers had measurable plasma d₆α-CEHC (Figure 2A), whereas all 20 participants had measurable amounts of d₆γ-CEHC (Figure 3). Baseline plasma d₆α-γ-CEHC concentrations for the smokers (2.6 ± 0.4 nmol/L) were less than one-half those of the nonsmokers (6.2 ± 2.0 nmol/L; \( P = 0.04 \)). Similarly, smokers’ plasma d₆γ-γ-CEHC concentrations (62.5 ± 8.8 nmol/L) were strikingly lower than those of the nonsmokers (104.4 ± 16.0 nmol/L; \( P = 0.017 \)).

After the initial supplement of 75 mg of each d₃-RRR- and d₆all-rac-α-tocopheryl acetate (=12 h after the first dose), labeled α-CEHCs were found in the plasma of smokers and nonsmokers. The labeled metabolites were detected throughout the supplementation period, but became undetectable in all participants by 5 d postsupplementation. The nonsmokers’ time of maximal concentration (tₘₐₓ) for labeled and unlabeled plasma α-CEHCs occurred at day –2. The smokers’ tₘₐₓ for labeled α-CEHCs peaked later in the time course (day 0), whereas their plasma d₃γ-α-CEHC remained relatively constant throughout the study period. The smokers’ d₃γ-α-CEHC maximal concentration (Cₘₐₓ; 8.6 ± 1.6 nmol/L) was less than one-third the nonsmokers’ Cₘₐₓ (26.1 ± 5.7; \( P = 0.004 \)). Similarly, the smokers’ d₆γ-α-CEHC Cₘₐₓ (20.2 ± 4.4 nmol/L) was significantly lower than the nonsmokers’ (50.0 ± 9.4 nmol/L; \( P = 0.007 \)). Moreover, the nonsmokers’ d₆γ-α-CEHC Cₘₐₓ was 2.3 times higher than their d₃γ-α-CEHC Cₘₐₓ (\( P = 0.001 \)), whereas this was 3.1 times higher in smokers (\( P = 0.002 \)).

The AUCs of labeled and unlabeled plasma α- and γ-CEHCs were all significantly lower among the smokers than among the nonsmokers (Figure 4). Specifically, smokers had ≈50% smaller plasma d₃γ- (\( P < 0.05 \)), d₃γ- (\( P < 0.05 \)), and d₃γ- (\( P < 0.05 \)) α-CEHC AUCs and an ≈40% smaller γ-CEHC AUC (\( P < 0.05 \)). In addition, the γ-CEHC AUC was >3 times the total α-CEHC AUC among both nonsmokers (\( P < 0.01 \)) and smokers (\( P < 0.01 \)). Taken together, these data suggest that smokers produced fewer CEHCs throughout the investigation.

**Plasma ratio of all-rac- to RRR-α-CEHC**

The efficacy or biopotency of all-rac-α-tocopheryl compared with RRR-α-tocopheryl is a subject of much debate (25). The plasma ratio of deuterated all-rac- to RRR-α-tocopheryl was previously reported to be 1:2, which is consistent with the preferential utilization of RRR-α-tocopherol (8). Therefore, we hypothesized that more all-rac would be available for metabolism and thus measured the ratio of plasma concentrations of d₆α-CEHC to d₃α-CEHC (d₆/d₃α-CEHC) to evaluate the relative metabolism of synthetic and natural α-tocopherols. The plasma d₆:d₃α-α-CEHC ratios of all participants are combined, because there were no significant differences between groups. As illustrated in Figure 5, more of the ingested d₆all-rac- than the d₃RRR-α-tocopheryl acetate was metabolized to α-CEHC, as noted by ratios of d₆:d₃α-CEHC that ranged between 2.9 and 4.7 throughout the entire study. Moreover, there was no significant change in the d₆:d₃α-CEHC ratio throughout the study, and the overall mean (±SE) was 3.9 ± 0.2. The greater d₆:d₃α-CEHC in plasma was consistent with the higher d₃:d₆α-tocopherol.
plasma ratio we previously observed (8). Therefore, all-rac-α-tocopherol appears to be more actively metabolized than is RRR-α-tocopherol regardless of smoking status, because a greater concentration of d₆-α-CEHC and a lower concentration of d₃-α-tocopherol was observed in the circulation of all participants.

Effect of α-tocopherol supplementation on γ-CEHC

Previously, we reported that 6 d of supplementation with deuterated α-tocopherol acetate significantly decreases plasma γ-CEHC concentrations by ~38% by day 0 in smokers and nonsmokers (8), but the effect of α-tocopherol supplementation on γ-CEHC was not explored. From the γ-CEHC time course (Figure 3), it appeared that the largest increase in γ-CEHC occurred between day −6 and day −5. Therefore, we performed correlations between plasma total α-tocopherol and γ-CEHC concentrations as well as between the change in total α-tocopherol and the change in γ-CEHC concentrations and observed no significant associations (P > 0.05). Similarly, we performed correlations for day 0 plasma total α-tocopherol and γ-tocopherol but observed no significant associations. Moreover, day 0 plasma γ-tocopherol and day 0 γ-CEHC were not significantly correlated (P > 0.05). Therefore, these data suggest that supplementation with α-tocopherol did not cause a significant reduction in plasma γ-tocopherol by increased γ-tocopherol metabolism to γ-CEHC.

Plasma α-CEHC as a possible marker of in vivo α-tocopherol status

Additional biomarkers for assessing α-tocopherol status are necessary (9) because plasma α-tocopherol concentrations are often similar between unsupplemented smokers and nonsmokers (26, 27). Therefore, we proposed to use plasma α-CEHC as a marker of α-tocopherol status and visualized the associations between α-tocopherol and α-CEHC, stratified by smoking status (Figure 6). We observed that d₆-, d₃-, and total α-tocopherol were associated with their respective α-CEHCs among smokers and nonsmokers (P < 0.0001). However, we observed no significant correlation for either smokers or nonsmokers between d₆-α-tocopherol and d₃-α-CEHC. To further estimate the main and interactive effects of α-tocopherol and smoking status on α-CEHC concentrations, we performed multiple linear regression by controlling for within-subject correlation (using SAS GENMOD). We observed that plasma d₆-, d₃-, and total α-tocopherol concentrations were highly associated with respective plasma d₆-, d₃-, and total α-CEHC concentrations among nonsmokers (P < 0.0001). Among smokers, the associations were also highly significant (d₆: P < 0.0001; d₃: P = 0.0003; total: P = 0.0158). Furthermore, multiple regression analysis indicated that there was a significant interactive effect of labeled α-tocopherol and smoking status on labeled α-CEHC concentrations (ie, the association between α-tocopherol and α-CEHC among smokers was different from that among nonsmokers). For example, among nonsmokers, the slope of the linear regression of d₆-α-CEHC on d₆-α-tocopherol was 3.78, whereas the slope among smokers was 1.94 (P = 0.0117). Thus, in the nonsmokers, d₆-α-CEHC increased 3.78 nmol/L for each 1-μmol/L increase in d₆-α-tocopherol, whereas in the smokers, d₆-α-CEHC increased only 1.94 nmol/L for the same plasma d₆-α-tocopherol increment. Similarly, nonsmokers compared with smokers had steeper slopes for d₃-(P = 0.0141) and total (P = 0.0170) CEHC.

Thus, given similar plasma α-tocopherol concentrations, smokers metabolized ~50% less α-tocopherol to α-CEHC than did nonsmokers.

DISCUSSION

In the present investigation, we showed that smokers had lower plasma concentrations of labeled and unlabeled α-CEHCs after supplementation with deuterium-labeled α-tocopherol acetate (75 mg each d₆-RRR- and d₆-all-rac-α-tocopherol acetate). The 6-d supplementation period was sufficient for smokers and nonsmokers to achieve plasma labeled and total α-tocopherol concentrations that were not significantly different on day 0 (8). However, plasma labeled and unlabeled α-CEHCs in smokers were significantly lower than in nonsmokers during the 10 d these labeled materials were detectable (Figure 2).

For this investigation, we used LC-MS to take advantage of the high selectively enhanced analyze specificity and low limit of detection in identifying unlabeled and deuterium-labeled plasma CEHCs. Importantly, the use of LC-MS enabled the determination of baseline unlabeled α-CEHCs that were comparable with values previously reported in unsupplemented individuals (14). Likewise, supplementation with deuterium-labeled α-tocopherol acetate resulted in total α-CEHC (d₆- + d₃- + d₆-α-CEHC) concentrations that were comparable with values reported from α-tocopherol–supplemented individuals (19). Therefore, LC-MS can be successfully used to evaluate circulating CEHC concentrations. In addition, the use of this technology proved important because plasma CEHCs (nmol/L) were present at substantially lower concentrations than were plasma tocopherols (μmol/L), and LC/MS, unlike traditional HPLC detection techniques, easily enables the discrimination of labeled CEHCs from unlabeled CEHCs.

Before supplementation, smokers had plasma d₆-α-CEHC and d₆-γ-CEHC concentrations that were ~60% and ~40% lower than those of the nonsmokers, respectively, despite plasma α- and γ-tocopherol concentrations that were not significantly different. About 12 h (day −5) after the first dose of d₆-RRR- and d₆-all-rac-α-tocopherol acetate, all subjects had detectable plasma d₆- and d₆-α-CEHCs (Figure 2). However, beginning on day −5, these deuterated α-CEHCs were consistently lower among the smokers. This finding is further emphasized by d₆- and d₆-α-CEHC AUCs (day −5 to day 5) that were ~50% lower in smokers than in nonsmokers (Figure 4). In addition, smokers’ plasma d₆- and d₆-α-CEHC Cmax values were 67% and 59% lower than the nonsmokers’, respectively. These findings are surprising in that d₆- and d₆-α-tocopherol concentrations were not significantly different between smokers and nonsmokers during the supplementation period (8). Overall, these findings indicate that smokers’ plasma α-CEHC responses to deuterated α-tocopherol supplementation were depressed in that smokers produced less d₆- and d₆-α-CEHC given the same α-tocopherol acetate dose, a similar plasma deuterated α-tocopherol response, and similar dietary intakes of α-tocopherol (~5 mg/d) (8).

To evaluate whether synthetic and naturally occurring α-tocopherol were metabolized similarly, we evaluated plasma d₆/d₃-CEHC ratios. Strikingly, we observed that more of the ingested synthetic d₆-all-rac-α-tocopherol acetate was metabolized to d₆-α-CEHC (Figure 5). In fact, the d₆/d₆-α-CEHC ratio ranged between ~2.9 and 4.7 throughout the investigation. Collectively, these findings suggest that synthetic α-tocopherol
FIGURE 6. Correlations between \( \alpha \)-tocopherols (\( \alpha \)-Ts) and \( \alpha \)-carboxyethyl-hydroxycromans (\( \alpha \)-CEHCs) in nonsmokers and smokers (\( n = 10 \) per group). Plasma \( \alpha \)-CEHC as a marker of \( \alpha \)-tocopherol status was evaluated by visualizing the associations between \( \alpha \)-tocopherol and \( \alpha \)-CEHC, stratified by smoking status for all plasma samples obtained from day 5 to day 5 by multiple linear regression. During this period, \( d_6 \), \( d_3 \), and total (\( d_0 \) + \( d_3 \) + \( d_6 \)) \( \alpha \)-tocopherols were associated with the respective \( \alpha \)-CEHCs among smokers and nonsmokers. However, no significant correlation was observed between \( d_0 \)-\( \alpha \)-tocopherol and \( d_0 \)-\( \alpha \)-CEHC in either smokers or nonsmokers. By multiple linear regression controlled for within-subject correlation, plasma \( d_3 \), \( d_6 \), and total \( \alpha \)-tocopherol concentrations were significantly associated with the respective plasma \( d_3 \), \( d_6 \), and total \( \alpha \)-CEHC concentrations among nonsmokers (\( P < 0.0001 \) for each association). Among smokers, these associations were also highly significant (\( d_3 \): \( P < 0.0001 \); \( d_6 \): \( P = 0.0003 \); total: \( P = 0.0158 \)). Furthermore, there were significant interactive effects of \( d_6 \)-\( \alpha \)-tocopherol and smoking status on \( d_6 \)-\( \alpha \)-CEHC (\( P = 0.0117 \)) as well as of \( d_3 \)-\( \alpha \)-tocopherol and smoking status on \( d_3 \)-\( \alpha \)-CEHC. The labeled \( \alpha \)-CEHC responses were \( \approx 50\% \) lower in the smokers than in the nonsmokers given a similar plasma concentration of labeled \( \alpha \)-tocopherol.
preparations are more actively metabolized, which is consistent with the view that vitamin E forms that are not maintained in plasma are subject to increased catabolism and excretion.

We previously reported (8) that smokers have a faster \( \alpha \)-tocopherol fractional disappearance rate than do nonsmokers, which is consistent with an in vivo antioxidant function of \( \alpha \)-tocopherol. Furthermore, at the end of the trial (day 17), smokers had significantly lower plasma \( d_1 \) and \( d_6 \)-\( \alpha \)-tocopherols and lower urinary \( d_1 \) and \( d_6 \)-\( \alpha \)-CEHCs. From those observations, we speculated that smokers likely had lower \( \alpha \)-CEHCs as the result of less \( \alpha \)-tocopherol available for metabolism. However, during the supplementation period (day −6 to day 0), plasma \( \alpha \)-tocopherols were not significantly different between the groups; nonetheless, the analysis presented here shows that during the same interval, smokers had lower plasma \( \alpha \)-CEHC concentrations. Therefore, plasma \( \alpha \)-tocopherol concentrations are unlikely to be the only factors that regulate the metabolism of \( \alpha \)-tocopherol to \( \alpha \)-CEHC, and these data suggest that the liver tocopherol concentration is critical.

Recently, Jeanes et al (28) reported that smokers have lower lymphocyte and platelet \( \alpha \)-tocopherol concentrations than do nonsmokers despite having plasma \( \alpha \)-tocopherol concentrations that are not significantly different. Therefore, one possibility for the smokers’ lower plasma \( \alpha \) and \( \gamma \)-CEHCs is that the \( \alpha \)-tocopheryl acetate supplementation period enabled tocopherol-containing tissues to be repleted. Subsequently, the hepatic \( \alpha \)-tocopherol transfer protein’s binding capacity for \( \alpha \)-tocopherol would be exceeded, and excess \( \alpha \)-tocopherol would accumulate in the liver and thus be available for the production of \( \alpha \)-CEHC. This view is consistent with that of Schultz et al (10), who suggested that \( \alpha \)-CEHC excretion is an indicator of optimal \( \alpha \)-tocopherol status and a saturated binding capacity. However, as an alternative or complementary possibility, it is also feasible that cigarette smoking dysregulated P450-mediated metabolism of tocopherols to CEHCs. Metabolism of tocopherols occurs via a cytochrome P450 of the 3A type (11, 29) or 4F type (13). In support of the CYP3A type, tocopherol was shown to prevent acetaminophen-induced CYP3A4 degradation in HepG2 cells (30). Furthermore, treatment of HepG2/C3A cells with sesamin (a known inhibitor of CYP3A) resulted in lower production of \( \gamma \)-CEHC (29). Cigarette smoke is known to induce certain cytochrome P450s, such as human CYP1A (31) and mouse CYP2E1 (32), whereas CYP3A protein levels are reportedly lower in smokers than in nonsmokers (33). Therefore, if a CYP3A is involved in \( \alpha \)-tocopherol metabolism and smoking decreases CYP3A protein levels, then it would be expected that smokers would have a lower rate of \( \alpha \)-tocopherol metabolism and thus lower plasma \( \alpha \) and \( \gamma \)-CEHC concentrations. Clearly, the molecular pathways involved in \( \alpha \)-tocopherol metabolism require additional investigations with more invasive models of oxidative stress to discern whether smoking decreases P450-mediated tocopherol metabolism or directly decreases tocopherols such that less tocopherol is available for P450-mediated metabolism.

In the United States, only 8% of men and 2.4% of women consume diets that meet the estimated average requirement for \( \alpha \)-tocopherol (34). Therefore, establishing a reliable biomarker for \( \alpha \)-tocopherol status is essential. In this investigation, we attempted to use \( \alpha \)-CEHC as a marker for \( \alpha \)-tocopherol status. However, given that all-rac-\( \alpha \)-tocopherol is more actively metabolized and smokers appear to metabolize less \( \alpha \)-tocopherol than do nonsmokers, it is unclear whether \( \alpha \)-CEHC concentrations can be validated as a suitable biomarker for \( \alpha \)-tocopherol status. Moreover, under normal dietary conditions, plasma \( \alpha \)-CEHC is minimal (in the nmol/L range) or undetectable unless individuals surpass a threshold (yet to be defined) that activates P450-mediated metabolism. Therefore, measurement of \( \alpha \)-CEHC might actually only represent excess \( \alpha \)-tocopherol rather than be a suitable marker for those with low or marginally low plasma \( \alpha \)-tocopherol. This is exemplified by the fact that at baseline, 5 of 20 participants had undetectable \( d_0 \)-\( \alpha \)-CEHC and, thus, may have had suboptimal \( \alpha \)-tocopherol status.

Additional work is clearly warranted to better understand the molecular mechanisms responsible for tocopherol metabolism and the effect that cigarette smoking or other oxidative stresses have on its regulation. Furthermore, investigations regarding \( \alpha \)-tocopherol liver trafficking should be considered, because we observed a maximal peak in plasma \( \alpha \)-CEHCs at day −2 that sharply declined (Figure 2) despite \( \alpha \)-tocopherol supplementation for 2 additional days. The fact that \( \alpha \)-CEHC did not remain elevated or continue to rise during the \( \alpha \)-tocopherol supplementation period suggests that alternative pathways, such as biliary \( \alpha \)-tocopherol excretion (15, 35, 36), may be activated to dispose of excess hepatic \( \alpha \)-tocopherol.

We express special thanks to the study participants for their cooperation throughout the investigation.

RSB, TMB, and MGT participated in the study design, data collection, and analyses and wrote the initial draft of the manuscript. JL assisted with the statistical analysis and participated in the editing and review of the manuscript. SWL participated in the sample analysis for CEHCs and contributed to the editing and review of the manuscript. None of the authors had a known conflict of interest.

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High prevalence of vitamin D deficiency among pregnant women and their newborns in northern India

Alok Sachan, Renu Gupta, Vinita Das, Anjoo Agarwal, Pradeep K Awasthi, and Vijayalakshmi Bhatia

ABSTRACT

Background: Vitamin D deficiency is prevalent in India, a finding that is unexpected in a tropical country with abundant sunshine. Vitamin D deficiency during pregnancy has important implications for the newborn and infant. There are few data from India about the prevalence of hypovitaminosis D in pregnancy and in the newborn.

Objective: Our aim was to determine the prevalence of osteomalacia and hypovitaminosis D in pregnancy and in cord blood and to correlate maternal 25-hydroxyvitamin D [25(OH)D] status with sun exposure, daily calcium intake (dietary plus supplemental), and intact parathyroid hormone (PTH) concentrations.

Design: Serum calcium, inorganic phosphorus, 25(OH)D, heat-labile alkaline phosphatase, and PTH were studied in 207 urban and rural pregnant subjects at term. Alkaline phosphatase and 25(OH)D were measured in the cord blood of 117 newborns.

Results: Mean maternal serum 25(OH)D was 14 ± 9.3 ng/mL, and cord blood 25(OH)D was 8.4 ± 5.7 ng/mL. PTH rose above the normal range when 25(OH)D was <22.5 ng/mL. Eighty-four percent of women (84.3% of urban and 83.6% of rural women) had 25(OH)D values below that cutoff. Twenty percent of the subjects had elevated alkaline phosphatase (17% of urban and 7% of rural subjects). Calcium intake was uniformly low, although higher in urban (842 ± 459 mg/d) than in rural (549 ± 404 mg/d) subjects (P < 0.001). Maternal serum 25(OH)D correlated positively with cord blood 25(OH)D (r = 0.79, P < 0.001) and negatively with PTH (r = −0.35, P < 0.001).

Conclusion: We observed a high prevalence of physiologically significant hypovitaminosis D among pregnant women and their newborns, the magnitude of which warrants public health intervention. Am J Clin Nutr 2005;81:1060–4.

KEY WORDS Vitamin D, pregnancy, osteomalacia, parathyroid hormone, newborn, sunlight, dietary calcium

INTRODUCTION

Vitamin D deficiency is unexpected in a tropical country such as India, where there is abundant overhead sun for most or all of the year. Nevertheless, hypovitaminosis D, resulting in severe osteomalacia, has been observed in adolescents in India (1). This paradox may be partly explained by the many prevalent social and cultural practices in India that preclude adequate exposure of adolescent girls and young women to sunshine. Revealing clothing is frowned on in traditional Indian households, both rural and urban. Newly married females are expected to cover themselves even more and are discouraged from outdoor activity. Increasing urbanization that results in poor outdoor activity and greater pollution, coupled with skin pigment, may further compound this problem (2).

Furthermore, milk, the primary source of calcium, is an expensive food in India. Deficient calcium intake has been shown to be the cause in a large proportion of childhood rickets in India (3) and other tropical countries (4, 5) and to contribute to adolescent osteomalacia (1, 3). Dietary calcium replenishment produced healing of rickets independent of vitamin D in those rickets patients with normal serum 25-hydroxyvitamin D [25(OH)D] concentrations (3, 4). Experimental studies in a rat model showed that dietary calcium deficiency caused secondary vitamin D deficiency and that calcium replenishment improved serum 25(OH)D concentrations (6). It is possible that the same mechanism may be active in human calcium-deficiency rickets or osteomalacia.

In a population that already has a high prevalence of vitamin D deficiency and poor dietary calcium intake, the problem is likely to worsen during pregnancy because of the active transplacental transport of calcium to the developing fetus. Hypovitaminosis D during pregnancy has important consequences for the newborn, including fetal hypovitaminosis D, neonatal rickets and tetany, and infantile rickets (7, 8). Rickets during infancy has been associated with higher prevalence of lower respiratory tract infections (9), the largest cause of infant mortality in India.

There are few data on serum 25(OH)D concentration and the prevalence of osteomalacia among pregnant women from India (10, 11). This study was undertaken to determine the prevalence of clinical or biochemical osteomalacia and maternal and fetal hypovitaminosis D among urban and rural northern Indian women and to study the correlation of those prevalences with calcium intake, sun exposure, serum 25(OH)D, and plasma intact parathyroid hormone (PTH).

SUBJECTS AND METHODS

Subjects

Pregnant women were recruited from Queen Mary’s Hospital, King George Medical University, Lucknow (lat 26.8°N), which

1 From the Department of Endocrinology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India (AS, PKA, and VB), and Queen Mary’s Hospital, King George’s Medical University, Lucknow, India (RG, VD, and AA).

2 Reprints not available. Address correspondence to V Bhatia, Department of Endocrinology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Rae-bareli Road, Lucknow 226014, India. E-mail: vbhatia@sgpgi.ac.in.

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caters to predominantly low and middle socioeconomic groups. The hospital serves both women who have never had antenatal care, who are predominantly from the villages surrounding the city of Lucknow, and women who have had regular antenatal care at the hospital itself, who are predominantly urban women. We attempted to recruit all women with a full-term live pregnancy who presented to the hospital in a 3-mo period from September to November 2002 and their infants. Exclusion criteria were chronic liver disease, renal disease, or treatment with antitubercular or antiepileptic drugs in the previous 3 mo. Because of a shortage of labor room staff, we were able to register only 207 of the 572 women who had term live newborns during the 3-mo study period. One hundred fifty-seven subjects were Hindu, and 50 were Muslim. Of the Muslim subjects, 29 women from both the urban and rural areas practiced purdah, in which the veil covers the whole body except hands and face.

Detailed history and examination were performed with special regard to current and past pregnancies and labor, socioeconomic status, and clinical features suggestive of osteomalacia (eg, proximal muscle weakness, bone pain, tenderness, or fractures) or past rickets. Daily intake of dietary calcium and vitamin D was calculated from a food-frequency questionnaire. The food-frequency questionnaire was validated for calcium in a sample of 30 subjects against a 5-d diet record (D Pandey, unpublished observations, 2004). A strong correlation of dietary calcium estimation was observed between the food-frequency questionnaire and the diet record ($r = 0.653$, $P < 0.001$). Any supplemental calcium intake in the current pregnancy was also noted. Daily sun exposure was assessed by taking a detailed history of the daily routine separately during summer and winter seasons and of the type of clothing worn. Sunshine exposure was calculated as hours of exposure/d × percentage of body surface area (BSA) exposed. Birth weight, crown-heel length, largest diameter of anterior fontanelle, and head circumference of newborns were examined.

Oral informed consent was obtained from all subjects. Approval from the institutional ethics committee was obtained.

Biochemical analysis

Maternal blood was collected in the nonfasting condition before labor and immediately transported on ice to the Sanjay Gandhi Institute for assay within 24 h for serum alkaline phosphatase (AP), calcium, albumin, and phosphorus. Sera were stored at $-70 \degree C$ for future analysis of serum 25(OH)D and PTH. Cord blood samples ($n = 117$) were similarly processed for AP activity and serum 25(OH)D.

Serum total calcium, albumin, and inorganic phosphorus were analyzed spectrophotometrically (Sigma Diagnostics, St Louis, MO). Serum calcium was corrected for serum albumin. Serum AP was measured spectrophotometrically (Boehringer Mannheim, Mannheim, Germany). To exclude placental isoenzyme (stable after heating for 15 min at 56 \degree C), heat-labile AP (HLAP) was analyzed (12). The normal upper limit for maternal HLAP was taken as that for total AP in our laboratory for an adult population (125 U/L), and the normal upper limit for cord blood AP was taken as 165 U/L (13). Serum 25(OH)D was assayed by using a commercial radioimmunoassay kit (Diasorin, Stillwater, MN). The sensitivity of this assay is 1.5 ng/mL, and the total imprecision CV is 8.2% at 22.7 ng/mL. Although the reference range given by the manufacturer of the assay is 9–38 ng/mL, those values represent a small number of subjects living in temperate latitudes and do not necessarily represent a true normal range for 25(OH)D. On the basis of physiologic correlates such as PTH, that range is more likely to be 20–80 ng/mL (14–17). The normal range of cord blood 25(OH)D was similarly taken as 20–80 ng/mL. Plasma PTH assay was performed by using a commercial immunoradiometric assay kit (normal range: 9–55 pg/mL; Diagnostic Systems Laboratories, Webster, TX). The sensitivity of this assay is 6 pg/mL, and the interassay CV is 10.5%.

Statistical analysis

Data are presented as mean (±SD). Statistical analysis was conducted by using SPSS FOR WINDOWS software (version 9.0; SPSS, Chicago, IL). Proportions were compared by using the chi-square test. Group means were compared by using Student’s $t$ test. Nonparametric data were log transformed and compared by using Student’s $t$ test. Correlations were studied by using Spearman’s correlation coefficient. To ascertain the 25(OH)D concentration below which PTH rose above the normal range, a linear regression analysis was performed. All complete pairs of values were used to derive a cutoff of 25(OH)D. Significance at $P \leq 0.05$ was taken for two-sided tests.

RESULTS

No difference between the subjects registered and those not registered was observed in age, weight at term, or religion (Table 1). However, the registered subjects had significantly lower parity and their newborns had significantly higher birth weight than did the nonregistered subjects and their newborns. None of the subjects had clinical evidence of osteomalacia, as defined by proximal muscle weakness and bony pains or tenderness. Biochemical osteomalacia (HLAP $>$125 U/L) was present in 29 subjects (14%). Subjects with biochemical osteomalacia had lower serum inorganic phosphorus and higher PTH than did women with normal HLAP (Table 2). However, maternal serum 25(OH)D, dietary calcium intake, and cord blood 25(OH)D did not differ significantly between the groups.

Maternal serum 25(OH)D $<$10 ng/mL was found in 88 women (42.5%), whereas 138 women (66.7%) had values $<$15 ng/mL. Plasma PTH was significantly higher (125 ± 153 and 51 ± 39 pg/mL, respectively; $P < 0.001$) and cord blood 25(OH)D was significantly lower (5.2 ± 3.0 and 11.8 ± 5.9 ng/mL, respectively; $P < 0.001$) in mothers with 25(OH)D concentrations $<$10 ng/mL than in mothers with 25(OH)D concentrations $>$10 ng/mL.

**TABLE 1**

Clinical characteristics of registered and not registered subjects

<table>
<thead>
<tr>
<th></th>
<th>Registered ($n = 207$)</th>
<th>Not registered ($n = 365$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>24.0 ± 4.1$^1$</td>
<td>24.7 ± 5.1$^1$</td>
</tr>
<tr>
<td>Weight at term (kg)</td>
<td>55.1 ± 6.5</td>
<td>52.5 ± 4.3</td>
</tr>
<tr>
<td>Parity</td>
<td>1.1 ± 1.2</td>
<td>1.8 ± 1.1$^2$</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>2.9 ± 1.7</td>
<td>2.7 ± 2.0$^2$</td>
</tr>
<tr>
<td>Hindu/Muslim</td>
<td>157/50</td>
<td>267/98</td>
</tr>
</tbody>
</table>

$^1$± SD (all such values).  
$^2$Significantly different from registered subjects, $P < 0.001$ (Student’s $t$ test).
TABLE 2
Differences in biochemical indexes and daily calcium intake among subjects with and without biochemical osteomalacia

<table>
<thead>
<tr>
<th></th>
<th>Subjects with osteomalacia (n = 29)</th>
<th>Subjects without osteomalacia (n = 178)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected serum calcium (mg/dL)</td>
<td>9.4 ± 0.6^2</td>
<td>9.4 ± 0.7</td>
</tr>
<tr>
<td>Serum phosphorus (mg/dL)</td>
<td>3.6 ± 1.2</td>
<td>4.2 ± 1.7^3</td>
</tr>
<tr>
<td>Serum 25(OH)D (ng/mL)^4</td>
<td>12.1 ± 8.0</td>
<td>14.3 ± 9.5</td>
</tr>
<tr>
<td>Maternal hypovitaminosis D [n (%)]^5</td>
<td>26 (89.7)</td>
<td>148 (83.1)</td>
</tr>
<tr>
<td>Serum PTH (pg/mL)^6</td>
<td>127 ± 180</td>
<td>74 ± 89^7</td>
</tr>
<tr>
<td>Cord blood AP (U/L)^7</td>
<td>172 ± 200</td>
<td>114 ± 61</td>
</tr>
<tr>
<td>Cord blood 25(OH)D (ng/mL)^8</td>
<td>8.1 ± 7.4</td>
<td>8.5 ± 5.4</td>
</tr>
<tr>
<td>Daily calcium intake (mg/d)^9</td>
<td>813 ± 435</td>
<td>737 ± 466</td>
</tr>
<tr>
<td>Daily calcium intake &lt;RDA [n (%)]^10</td>
<td>22 (75.8)</td>
<td>138 (77.5)</td>
</tr>
<tr>
<td>Sun exposure score over past 3 mo (h/d)</td>
<td>4.9 ± 4.5</td>
<td>6.2 ± 6.0</td>
</tr>
</tbody>
</table>

^2,^5,^6 Significant different from subjects with osteomalacia (Student’s t test after log transformation of data and of proportions with chi-square test): ^2P < 0.05; ^5P < 0.005. ^4,^7 Hypovitaminosis D = < 22.5 ng 25(OH)D/mL. ^8 n = 157 (with osteomalacia, n = 24; without osteomalacia, n = 133). Normal = 9–55 pg/mL. ^9 Normal = < 165 U/L. ^10 Dietary plus supplemental calcium intake.

Maternal serum 25(OH)D showed a strong positive correlation with cord blood 25(OH)D (r = 0.79, P < 0.001) and a moderate negative correlation with maternal plasma PTH (r = −0.35, P < 0.001) (Figure 1). The regression equation between serum 25(OH)D and plasma PTH yielded a 25(OH)D value of 22.5 ng/mL, below which PTH rose beyond the upper limit of normal. Eighty-four percent of women had 25(OH)D concentrations <22.5 ng/mL. A weak correlation also existed between maternal HLAP and cord blood AP (r = 0.19, P < 0.05). Maternal serum 25(OH)D did not correlate with HLAP, sun exposure, or daily calcium intake.

A comparison of women of urban and rural backgrounds is shown in Table 3. Sun exposure was significantly lower in urban subjects than in rural subjects in the last trimester of pregnancy (urban: 4.1 ± 3.2 h/d × %BSA exposed; rural: 9.7 ± 8.1 h/d × %BSA exposed; P < 0.001) as well as over the previous year (urban: 7.5 ± 5.6 h/d × %BSA exposed; rural: 11.6 ± 8.4 h/d × %BSA exposed; P = 0.005). Despite this finding, the mean serum 25(OH)D concentration in urban women did not differ significantly from that in rural women (urban: 14.0 ± 9.5 ng/mL; rural: 14.1 ± 8.9 ng/mL; NS). In contrast, the dietary calcium intake was significantly lower in rural than in urban women (549 ± 404 and 842 ± 459 mg/d, respectively; P < 0.001). Mean maternal PTH and HLAP were significantly higher in urban women. Total daily calcium intake, mean HLAP, 25(OH)D, and PTH did not differ significantly between women practicing purdah and women not practicing purdah.

**TABLE 3**
Clinical characteristics and biochemical indexes of urban and rural women

<table>
<thead>
<tr>
<th></th>
<th>Urban women (n = 140)</th>
<th>Rural women (n = 67)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sun exposure score over past 3 mo (h/d × %BSA exposed)</td>
<td>4.1 ± 3.2^2</td>
<td>9.7 ± 8.1^4</td>
</tr>
<tr>
<td>Sun exposure score over past 1 y (h/d × %BSA exposed)</td>
<td>7.5 ± 5.6</td>
<td>11.6 ± 8.4^4</td>
</tr>
<tr>
<td>Daily calcium intake (mg/d)^5</td>
<td>842 ± 459</td>
<td>549 ± 40^4</td>
</tr>
<tr>
<td>Daily calcium intake &lt;RDA [n (%)]^6^7</td>
<td>101 (72)</td>
<td>59 (88)^7</td>
</tr>
<tr>
<td>Daily vitamin D intake (IU/d)</td>
<td>16.4 ± 7.4</td>
<td>16.5 ± 7.7</td>
</tr>
<tr>
<td>HLAP (U/L)^8</td>
<td>87 ± 60</td>
<td>73 ± 31</td>
</tr>
<tr>
<td>Elevated HLAP [n (%)]^9</td>
<td>24 (17)</td>
<td>5 (7)</td>
</tr>
<tr>
<td>Serum 25(OH)D (ng/mL)^10</td>
<td>14.0 ± 9.5</td>
<td>14.1 ± 8.9</td>
</tr>
<tr>
<td>Maternal hypovitaminosis D [n (%)]^11</td>
<td>118 (84)</td>
<td>56 (84)</td>
</tr>
<tr>
<td>Maternal PTH (pg/mL)^12</td>
<td>94 ± 127</td>
<td>57 ± 49</td>
</tr>
</tbody>
</table>

^2,^4 SD (all such values). ^2,^5,^6,^7 Significantly different from urban women (Student’s t test after log transformation of data and of proportions with chi-square test): ^2P < 0.001, ^5P < 0.005, ^6P < 0.05. ^8 Dietary plus supplemental calcium intake.

**FIGURE 1.** Scatter plot showing relation of intact parathyroid hormone (PTH) and serum 25-hydroxyvitamin D (25(OH)D) in mothers’ blood (n = 157). Regression equation (by linear regression analysis): PTH = [−3.32 × 25(OH)D] + 129.76.

The mean cord blood 25(OH)D in neonates was low (8.4 ± 5.7 ng/mL). A large proportion of neonates (95.7%) had hypovitaminosis D [serum 25(OH)D <20 ng/mL]. Mean AP was 131...
DISCUSSION

Our study presents the first large body of data on serum 25(OH)D and PTH in pregnancy from a population not observing purdah in a tropical country. The most important finding in our study is the unexpectedly high prevalence of hypovitaminosis D among pregnant women. The physiologic relevance of the finding is substantiated by the negative correlation with PTH and the positive correlation with cord blood 25(OH)D. Hypovitaminosis D and osteomalacia among pregnant South Asian women have been widely reported (10, 11, 18–25). However, all studies but a few (ie, 10, 11, 22, 23) were from temperate regions such as the United Kingdom (18–21, 24) and Norway (25), where the already low availability of overhead sun is compounded for Asian women by poor outdoor activity, pigmented skin, and excessive clothing. Vitamin D deficiency has also been noted in pregnant women in tropical countries, but all studies were in Muslim populations, in whom the practice of purdah might have played an important role (22, 23, 26–29). The only study to comment on serum 25(OH)D concentrations in pregnant non-Muslim women living in the tropics is from New Delhi (11), where the mean concentration in summer in 25 women was 21.9 ± 10.7 nmol 25(OH)D/L (8.6 ± 4.28 ng/mL).

We expected to find a higher serum 25(OH)D concentration in the rural women in our study than in their urban counterparts, who had distinctly poorer sun exposure. However, the results were contrary to expectation, with urban and rural women having equally low mean serum concentrations and equally high prevalence of the deficiency. The explanation could lie in the prolonged deficiency of dietary calcium intake among poorer parts of India (where most of the rural women in our study lived), because of the expensive nature of milk and milk products. Dietary calcium deficiency has been shown to lead to secondary vitamin D deficiency in rats (6). Similar findings are also suggested in studies on humans (3, 4). Our own studies among children with rickets and adolescent girls with rickets or osteomalacia who were from a lower socioeconomic population showed the average daily dietary calcium intake in these 2 groups to be 282 mg and 305 mg, respectively (1, 3). The higher intake of dietary calcium in the women in our study is likely to have been short-lived and attributable to the social custom of providing extra milk to pregnant and lactating women. Further studies are needed to document direct evidence of improvement in serum 25(OH)D with calcium supplementation in large numbers of subjects in our region.

Exactly how much sun exposure is needed for healthy people to maintain normal serum 25(OH)D is not clear. It would, of course, depend on latitude, season, skin pigmentation, and age. On the basis of his own studies, Holick (30) recommended that suberythemal exposure of face, arms, and hands (ie, ≈22% BSA) ≈3 times a week is probably sufficient for elderly people living in a temperate climate to maintain serum 25(OH)D at 20 ng/mL. It would be expected, then, that a similar amount of vitamin D should form in the skin of the women in our study, who were younger and lived in a more tropical latitude, and who exposed =11% of their BSA to sun for 1 h/d. In addition to the possible contribution of darker skin pigment and prolonged low intake of dietary calcium, the high amount of atmospheric pollution extant in Indian cities, including Lucknow, could be an important factor (2, 31).

The cutoff of 10 ng 25(OH)D/mL, which we used a priori for defining hypovitaminosis D, is conservative. The availability of simultaneous PTH and serum 25(OH)D allowed us to examine the relation between these 2 hormones. Most investigators now suggest higher values of 25(OH)D, eg, 15–30 ng/mL, as the cutoff below which PTH starts to rise sharply (14–16, 32). Investigators who used other surrogate markers such as intestinal calcium absorption and bone mineral density suggested 25(OH)D concentrations as high as 98 nmol/L (39.2 ng/mL) to define normalcy (33, 34). In our study also, the corresponding 25(OH)D value was 22.5 ng/mL. Accordingly, 84% of the women in our study would be declared vitamin D–deficient.

Cord blood 25(OH)D strongly correlated with maternal values, which is in keeping with reports in the literature (19, 35–37). The cutoff for hypovitaminosis D in neonates is still being debated. No evidence suggests that neonatal 25(OH)D concentrations are different from those in adults. Zeghoud et al (36) found neonatal 25(OH)D concentrations <30 nmol/L (12 ng/mL) to be associated with elevated PTH, and they proposed that concentration as the cutoff for diagnosing hypovitaminosis D in the newborn. We were unable to study the status of neonatal calcium and PTH. However, on the basis of what is known in the literature, we can conclude that a large proportion of our newborns have 25(OH)D concentrations that will predispose them to neonatal hypocalcemia and infantile rickets and to the attendant morbidity (8, 38, 39).

In the current study, 14% of the mothers had elevated HLAP (which indicated biochemical osteomalacia), as did 14% of the newborns. Although none of these women had clinical features suggestive of osteomalacia, the biochemical profile (ie, low serum phosphorus and elevated PTH) is that typically seen in osteomalacia. Brooke et al (19) reported elevation of HLAP in 20% of Asian subjects from the United Kingdom with serum 25(OH)D concentrations <25 nmol/L (10 ng/mL), whereas only 2% of those who had serum 25(OH)D concentrations >25 nmol/L had elevated HLAP. Rab and Baseer (22) from Pakistan reported elevated total AP in 26% of pregnant women. Daily vitamin D intake was low (88 ± 14 IU/d) in their subjects, but serum 25(OH)D was not measured. Marya et al (10) from India reported elevated HLAP in 13% and hypocalcemia in 44% of their pregnant subjects who were not receiving vitamin D supplementation, whereas none of the subjects supplemented with vitamin D (600 000 IU twice in the 7th and 8th mo of gestation) had elevated HLAP. That study also did not comment on serum 25(OH)D.

At present, vitamin D supplementation is not a part of antenatal care programs in India. The US National Academy of Sciences mentions 400 IU as the dietary reference intake for vitamin D during pregnancy. However, several investigators worldwide are arguing for revised higher guidelines for vitamin D allowance during pregnancy and lactation (40). So far, the concern expressed by those investigators is mainly for women in temperate climates, especially those with greater skin pigmentation, and for women living in tropical regions but observing purdah, such as those in the Middle East. On the basis of our results, we conclude
that such recommendations perhaps are also warranted for pregnant Indian women not practicing purdah, so that they may remain healthy and provide adequate vitamin D to their fetuses. The exact cause of or factors contributing to the occurrence of hypovitaminosis D in rural women in a tropical country remain to be elucidated in future studies.

We thank Diwa Pandey for sharing information on food-frequency questionnaire validation for calcium and Eshh Bhatia for helpful discussion. AS designed the study, collected and analyzed the data, and wrote the manuscript; RG collected the data and wrote the manuscript; VD designed the study and analyzed the data; AA designed the study and analyzed the data; PKA collected the data; VB designed the study, analyzed the data, and wrote the manuscript. None of the authors had a conflict of interest.

REFERENCES

Effects of chronic copper exposure during early life in rhesus monkeys¹–³

Magdalena Araya, Shannon L. Kelleher, Miguel A. Arredondo, Walter Sierralta, María Teresa Vial, Ricardo Uauy, and Bo Lönnerdal

ABSTRACT

Background: Whether infants regulate copper absorption and the potential effects of excess copper in early life remain poorly defined.

Objective: The objective of the study was to assess copper retention, liver copper content, and liver function in infant rhesus monkeys fed infant formula containing 6.6 mg Cu/L.

Design: From birth to 5 mo of age, infant rhesus monkeys were fed formula that was supplemented with copper (0.6 mg Cu/L; n = 5) or not supplemented (n = 4). In all animals, weight and crown-rump length (by anthropometry), hemoglobin, hematocrit, plasma ceruloplasmin activity, and zinc and copper concentrations were measured monthly (birth to 6 mo) and at 8 and 12 mo. When the animals were 1, 5, and 8 mo old, liver copper and metallothionein concentrations, liver histology (by light and electron microscopy), and the number of Kupffer cells were assessed, and ⁶⁷Cu retention was measured. Liver function was assessed by measurement of plasma alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, and alkaline phosphatase activities and protein, albumin, bilirubin, and blood urea nitrogen concentrations.

Results: ⁶⁷Cu retention was 19.2% and 10.9% after 1 and 5 mo of copper treatment, respectively, compared with ≈75% in controls at age 2 mo. At age 8 mo, ⁶⁷Cu retention was 22.9% in copper-treated animals and 31.5% in controls. Liver histology remained normal by light microscopy, with mild ultrastructural signs of cell damage at 5 mo. Liver copper concentration was 471, 1139, and 498 g Cu·kg⁻¹·d⁻¹ for 14 d showing that copper absorption, studied by using stable isotopes, was similarly high at 1 and 3 mo of age, which suggests either that young infants cannot down-regulate copper absorption as has been observed in adults (1), or that the dose or duration (or both) of the load used for the study was insufficient to alter copper homeostasis, and therefore the copper did not trigger a compensatory response (2). To address this question, we report here the effects of controlled, chronic (from birth to 5 mo of age) copper exposure in an infant rhesus monkey (Macaca mulatta) model, with the use of a copper load based on estimations of the copper intake needed to induce Indian childhood cirrhosis (9). In addition, long-term consequences of high neonatal copper intake were addressed by monitoring liver histology and function up to 8 mo of age, i.e., 3 mo after exposure to high copper had been discontinued.

Conclusions: No clinical evidence of copper toxicity was observed. Copper absorption was down-regulated; increases in liver copper content at ages 1 and 5 mo did not result in histologic damage. Ultrastructural changes at age 5 mo could signal early cellular damage.

KEY WORDS Copper load, rhesus monkey, neonate monkeys, copper absorption, liver

INTRODUCTION

Homeostatic mechanisms regulate copper absorption over a wide range of intakes in humans. Copper absorption in young adults measured by using ⁶⁵Cu retention, was found to change from 55.6% to 36.3% and to 12.4% when copper intake was 0.79, 1.68, and 7.53 mg/d, respectively (1). This suggests that copper absorption is dependent on copper intake over a wide range of dietary exposures. Yet the limits of homeostasis—specifically, the amount of copper exposure that can result in early, detectable adverse consequences—remain unclear. Clinical controlled studies in infants (2) and community-based intervention studies in apparently healthy adults exposed to 3–10 times the customary copper intake (up to 9 mg Cu/d) have not shown significant changes in traditional indicators of copper status (3–5).

Data regarding copper absorption and its regulation during early life are limited. The assumptions are that copper accumulates in fetal liver during the third trimester of pregnancy (6, 7) and that stored copper will meet infant copper requirements during the breastfeeding period, while milk copper concentration is low (8). However, the risk of excess copper exposure in young infants fed formulas prepared with water containing high concentrations of copper remains a concern among pediatricians living in areas where drinking water may contain more than a few (1–2) milligrams of copper per liter of water. Our group (2) reported results from infants exposed to 80 μg Cu·kg⁻¹·d⁻¹ for 14 d showing that copper absorption, studied by using stable isotopes, was similarly high at 1 and 3 mo of age, which suggests either that young infants cannot down-regulate copper absorption as has been observed in adults (1), or that the dose or duration (or both) of the load used for the study was insufficient to alter copper homeostasis, and therefore the copper did not trigger a compensatory response (2). To address this question, we report here the effects of controlled, chronic (from birth to 5 mo of age) copper exposure in an infant rhesus monkey (Macaca mulatta) model, with the use of a copper load based on estimations of the copper intake needed to induce Indian childhood cirrhosis (9). In addition, long-term consequences of high neonatal copper intake were addressed by monitoring liver histology and function up to 8 mo of age, i.e., 3 mo after exposure to high copper had been discontinued.

¹ From the Institute of Nutrition and Food Technology (INTA), University of Chile, Santiago, Chile (MA, MAA, WS, and RU); the Departments of Nutrition and Internal Medicine, University of California, Davis, Davis, CA (SLK and BL); Universidad de Anatomía Patológica, Hospital San Borja Arriarán, Santiago, Chile (MTV); and Public Health Nutrition, London School of Hygiene & Tropical Medicine, London, United Kingdom (RU).

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³ Address reprint requests to M Araya, Institute of Nutrition and Food Technology, Macul 5540, Santiago 11, Chile. E-mail: maraya@inta.cl.

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MATERIALS AND METHODS

Study design

This protocol was approved by the Animal Care and Resources Committee and the Radiation Use Authorization Committee at the University of California, Davis. Infant rhesus monkeys were obtained from the California National Primate Research Center (Davis, CA). Animals were kept indoors under the constant care of nursery and veterinary staff and were bottlefed ad libitum from birth to age 5 mo with a standard, commercially available infant formula (Enfamil; Mead Johnson Nutritionals, Evansville, IN; n = 4) containing 0.6 mg Cu/L (n = 4) or the same formula supplemented with CuSO4 (an additional 6.0 mg Cu/L) (n = 5). From birth to 1 mo, the animals were individually housed in polycarbonate isoltes with a surrogate mother (a terrycloth dummy); from 1 to 5 mo of age, 2 animals were housed in each stainless-steel cage. Food intake was monitored daily. Monthly anthropometric measures (weight and crown-rump length) and fasting (>2 h after last feeding) venous blood samples were taken. At ages 6, 8, and 12 mo (1, 3, and 6 mo after discontinuation of the copper load), blood samples were drawn, and the measurements were repeated. At ages 1, 5, and 8 mo, copper absorption was measured after radioisotope administration (67Cu), and a biopsy was performed of liver tissue from all animals unless otherwise stated.

Hematologic studies

Hemoglobin, hematocrit, and white blood cell differential (CD4/CD8) were quantified with an automated electronic cell counter (Baker 9010 Analyzer; Serono-Baker, Allentown, PA). Ceruloplasmin oxidase activity in plasma was assayed with the use of o-dianisidine dihydrochloride according to the method of Schosinsky et al (10).

Absorption studies

Copper retention was measured after radioisotope administration. Monkeys were fasted for 4 h before being fed the radioisotopically labeled formula [≈1 μCi 67Cu (Brookhaven National Laboratory, Brookhaven, NY)/3 mL diet] by orogastric intubation. Immediately after dosing, each animal was placed inside a whole-body counter (Institute of Toxicology and Environmental Health, University of California, Davis, Davis, CA) equipped with two 10 × 20–cm sodium iodide crystals and a multichannel analyzer (ND-66; Nuclear Data, Schaumburg, IL) for measurement of the amount of radioactivity administered. No food was given for 2 h after dosing. Radioactivity in the animals was recounted after 4 d to ascertain the amount of isotope retained. Whole-body copper retention was calculated as the amount of radioactivity retained after 4 d, and isotopic decay was taken into account.

Plasma mineral analysis

Samples were digested with 0.1% (by vol) ultrapure nitric acid as described by Clegg et al (11). Plasma copper and zinc concentrations were analyzed by flame atomic absorption spectrophotometry (Thermo Jarrell Ash, Franklin, MD). Bovine liver preparations were used as reference materials (Standard Reference Material 1577; National Institute of Standards and Technology, Gaithersburg, MD) to validate the mineral analyses.

Liver enzymes

Total protein, albumin, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, alkaline phosphatase, bilirubin, creatinine, and blood urea nitrogen were measured by the Veterinary Clinical Diagnostic Laboratory at the University of California, Davis, with the use of commercially available kits (Roche Diagnostics, Indianapolis, IN), and analyzed by using a Hitachi 7171 Clinical Chemistry Autoanalyzer (Roche Molecular Biochemicals, Indianapolis, IN).

Studies in biopsied liver tissue

Tissues for biopsy were obtained by suction of the liver with the use of a small (infant) needle, always in the right-side liver lobe and with the needle inserted in different directions at each insertin to avoid sampling only one area. The tissue samples were cut into pieces for light and electron microscopy and for measurement of tissue copper content. One piece was fixed in 4% formaldehyde for 8–12 h at room temperature and then washed twice in buffer and progressively dehydrated with graded ethanol and xylene. Tissue was embedded in paraffin, serially sectioned at 5 μm, and stained for hematoxylin and eosin and rhodamine, according to routine procedures. Specific monoclonal antibodies were used to assess metallothionein concentration and the number of Kupffer cells by immunohistochemical techniques. For Kupffer cell assessment, a semiquantitative scale was developed, defining 0 as absence of staining and +++ as the strongest staining observed. The number of Kupffer cells and apoptotic cells were expressed as the mean number of cells per 5 fields. Two observers (MTV and MA) performed all histologic evaluations unaware of the animal assessed; their evaluations showed >95% agreement. The third piece of tissue was snap-frozen and maintained at −70 °C until it was analyzed.

Electron microscopy studies

For transmission electron microscopy, tissue was fixed for 8–10 h in 4% paraformaldehyde plus 0.5% glutaraldehyde in phosphate buffer (0.1 mol/L; pH 7.4), at room temperature. After being washed 3 times in phosphate buffer, the tissue was dehydrated with graded ethanol and embedded in LR-Gold resin (EMS, Fort Washington, PA) as described previously (12). After peroxide-induced polymerization in cold, the resin blocks were cut in a Reichert ultramicrotome, the 70-nm thin sections were collected on formvar-coated 300 mesh grids (EMS) and then stained with uranyl acetate and lead citrate. The specimens were assessed with a CM100 electron microscope (Philips Electron Optics, Eindhoven, Netherlands) operating at 80 kV.

Measurement of copper content in biopsied liver tissue

The biopsied liver tissues were weighed and dried for 12 h at 200 °C. The tissue was processed according to the Association of Official Analytical Chemists (13). Briefly, the tissue was digested with a mix of ultrapure nitric acid (2 mL) and sulfuric acid (2 mL) and boiled in a micro-Kjeldahl digestion unit (Labconco Corp, Kansas City, MO) for 15 min. The digest was cooled, perchloric acid (2 mL) was added, and the mixture was boiled again until the sample was colorless and transparent. The digest was diluted with 10 mL demineralized and double-distilled water. The copper content was measured by using an atomic absorption spectrometer equipped with graphite furnace (SIMAA...
6100; Perkin Elmer, Shelton, CT). MR-CCHEN-002 (Venus antiqua) and Dolt-2 (Dogfish liver) preparations were used as reference materials to validate the mineral analyses.

**Statistical analysis**

Values are given as mean (±SD). Statistical analysis was performed by two-factor repeated-measures analysis of variance with a Bonferroni posttest or t test (for 67Cu retention, liver enzymes, and liver concentration) by using GRAPHPAD PRISM software (version 3.02; GraphPad, San Diego, CA). Main effects of copper treatment and the interaction between copper treatment and age were ascertained. Significance was set at P < 0.05.

**RESULTS**

The copper-supplemented formula was well accepted; food intake did not differ significantly between copper-treated and control animals (data not shown). There was a trend toward an age × copper treatment interaction for weight that favored the high copper group, but the difference was not significant (P = 0.06) (Figure 1). There was no significant age × copper treatment interaction for hemoglobin concentration, hematocrit, plasma copper concentration, or ceruloplasmin activity (Figure 1). The age × copper treatment interaction for plasma zinc concentration was significant (P < 0.05). Copper-supplemented animals had significantly lower plasma zinc concentrations than did control animals during the copper supplementation period; however, after discontinuation of copper supplementation, plasma zinc concentrations returned to normal (Figure 2). There was no significant effect of copper treatment on liver aminotransferases or alkaline phosphatase. Retention of 67Cu at ages 1 and 5 mo was 19.8 ± 2.6% and 10.9 ± 2.0%, respectively, in the copper-treated animals (n = 4 animals/group). We were unable to evaluate 67Cu retention in control animals at those ages because of the unavailability of 67Cu at the specific times (ie, the production of the short-lived isotope at the intended age did not coincide with the availability of control animals at the UC Davis Primate Center), but data from our previous studies showed that ≈75% of 67Cu is retained in control animals at ages 1 and 5 mo (14). At age 8 mo—ie, 3 mo after cessation of copper supplementation—there was no significant effect of prior copper treatment on 67Cu retention (control animals: 31.5 ± 13%; copper-treated animals: 22.9 ± 5.6%).

A limitation to this study is that liver tissue for only one biopsy was obtained from each control animal, and that sample was

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**FIGURE 1.** Mean (±SD) crown-rump length, weight, hemoglobin, and hematocrit during the first 12 mo of life in Macaca mulatta exposed (●; n = 5) or not exposed (○; n = 4) to 6 mg CuSO4/L. A trend was noted only for copper exposure and weight (P = 0.06).
obtained when the animals were 2 mo old; thus, comparison of experimental animals at ages 1 and 5 mo is made by contrasting those findings with these single samples of biopsied tissue. Architecture and histologic characteristics of copper-treated animals were normal as examined by light microscopy (Figure 3) and did not differ significantly from those observed in control animals at all ages. Signs of tissue scarring were not observed, which suggests that tissue samples for consecutive biopsies were obtained from different places. Rhodamine staining was negative in biopsied tissue obtained at age 1 mo; however, in that obtained at age 5 mo, fine, small cytoplasmic granules were seen in some areas, mainly around the central venules, in copper-treated animals (Table 1); in control animals, rhodamine staining was negative at all times. When the numbers of Kupffer cells seen in the biopsied tissue obtained at 2 mo (the control biopsies) and in the biopsied tissue obtained at ages 1 and 5 mo, the copper-treated animals showed 70% and 49% more Kupffer cells, respectively, than did the controls.

Positive metallothionein staining was found in both the cytoplasm and nuclei in controls and copper-treated animals at 1 mo, and the distribution was mainly around the central venule. Semiquantitative analysis of metallothionein staining classified copper-treated animals as (+ + +), whereas controls were (+). Pyknotic nuclei and apoptotic cells were few in all animals, but, at age 5 mo, there were twice as many apoptotic cells in copper-treated animals as in control animals. At age 8 mo, all indicators measured were normal, and none differed significantly between treated and untreated animals. After 1 mo of copper treatment, irregularly shaped nuclei containing condensed chromatin were often seen on electron microscopy. Abundant, polymorphic mitochondria ranged in size from 0.5 to 1 μm and displayed visible cristae. Hepatocytes contained numerous secondary lysosomes, which were filled with electron dense material. In many cells,
there were scanty glycogen rosettes and numerous small vesicles; in general, rough endoplasmic reticulum (RER) was prominent, but in cells that were rich in vesicles, the RER was somewhat distorted. At 5 mo, the cell ultrastructure of hepatocytes was normal in all animals, with well-defined mitochondria, regularly shaped nuclei, chromatin decondensation, abundant mitochondria of normal appearance with well-defined cristae, few secondary lysosomes, abundant glycogen rosettes, few or no vesicles, and profuse RER.

The limitation of obtaining only one liver tissue sample for biopsy from the control animals also applies to copper measurements in liver. After 1 mo of copper treatment, the liver copper concentration was 4711 μg/g dry tissue weight (Table 1); this value decreased to 1139 μg/g dry tissue weight at age 5 mo and then progressively decreased to 498 μg/g dry tissue weight at age 8 mo. Yet, at age 8 mo, or 3 mo after discontinuation of copper treatment, copper-treated animals still had significantly higher liver copper concentrations than did control animals. If measurements in experimental animals at ages 1, 5, and 8 mo are compared with the single measurement performed at age 2 mo in the control animals, the differences are significant (P = 0.001, P = 0.001, and P = 0.005, respectively).

**DISCUSSION**

Indicators of copper status such as serum copper and ceruloplasmin concentrations remain unchanged in human infants consuming different amounts of copper (15–17). We observed similar results in infant monkeys, which suggests that blood copper homeostasis is tightly regulated. However, the effects of copper on small intestine and liver, tissues that play a dominant role in copper homeostasis, show evidence of regulatory responses. Mechanisms that control intestinal copper absorption are immature during early neonatal life in rats (18), which potentially places younger animals at greater risk for adverse consequences of high copper exposure. Copper supplementation of suckling rat pups resulted in small-intestine copper concentration that was significantly higher than that in control rats, but plasma copper concentration was not affected (19). In addition, young copper-treated rats accumulated more hepatic copper, had more severe liver changes, and had higher serum liver enzyme activities than did adult rats (20), which further suggests that the ability to regulate copper homeostasis is age-dependent. In our infant monkeys, high copper intake during the first 5 mo of life was well tolerated and resulted in no evidence of clinical copper toxicity, whereas copper treatment did result in a progressive reduction in 67Cu retention, which suggests that copper retention is indeed down-regulated in infant primates when dietary copper intake is high. Previous studies in human infants fed 80 μg Cu/kg/d did not detect changes in copper retention between 1 and 3 mo of age (2), but copper exposure in the current study was ~75 times higher than that in the study conducted in human infants, and it more adequately reflects the copper intake of infants fed infant formula that has been reconstituted with copper-contaminated drinking water (9).

Copper treatment induced detectable changes in the liver (Table 1). At 1 mo, liver copper concentration was significantly higher in copper-treated infants than in controls (4711 and 250 μg/g dry tissue, respectively), and this difference was associated with a 70% increase in Kupffer cells, which suggests some form of stress or a trigger for inflammation. After birth and over the first months of life, these cells are thought to progressively increase in number, but no reference data are available from which to estimate the magnitude and the precise timing for this in humans or primates. Kupffer cells are the resident macrophages in the liver, and they are known to migrate rapidly and proliferate locally in response to various stimuli (21–23). Therefore, it is possible that the marked increase in the numbers of Kupffer cells found after 1 mo of copper treatment may be a tissue response to manage oxidative stress due to copper exposure rather than a result of age alone. However, these findings were not associated with evidence of hepatitis, which contrasts with observations made in Fisher rats by Fuentelahba et al (20), who found multifocal hepatitis and widespread single-cell necrosis in copper-treated rats. Detection of metallothionein was markedly increased, and electron microscopy corroborated that copper was indeed deposited in hepatocytes and that at least part of the copper was retained in lysosomes, as reported by others (20). The fact that not all measurements could be done exactly as planned represents a limitation of this study.

At age 5 mo in these monkeys, liver copper content remained high, whereas copper retention had decreased from 19.8 ± 2.6% to 10.9 ± 2.0%. Biliary excretion is immature at birth, and serum bile acid concentration is low as reflected by the low efficiency of intestinal fat absorption (24). Serum bile acid concentrations
increase after birth, and they remain significantly higher through the first 4 to 6 mo of life than they are in adults (25, 26). One can speculate that age-related improvement in biliary function may have contributed to the lower liver copper concentration we observed at age 5 mo than at age 1 mo, because bile is the primary route for copper excretion. After 5 mo of copper loading, there were no signs of hepatitis and no further increase in the number of Kupffer cells, but the proportion of cells in which apoptotic nuclei were observed was significantly greater at age 5 mo than at age 1 mo. This observation agrees with those of others in rats (20) and may reflect the duration of copper treatment. In contrast to the high copper concentration measured in the liver, only a light (fine, granular) positive rhodamine staining was detected in the liver, which suggests that copper may be sequestered in a form or compartment that does not react with rhodamine. It is interesting that, in children with biliary atresia, Wilson disease, and Indian childhood cirrhosis (in which excess copper is deposited in the liver), this staining is intensely positive (9, 27). It is particularly interesting that Wilson disease results from a mutation in the \textit{ATP7B} gene, which encodes a copper-specific ATPase (28, 29). The normal \textit{ATP7B} gene product (Atp7B) is localized to the trans-Golgi apparatus and, in cultured hepatocytes, relocates to a vesicular compartment to either sequester copper or facilitate copper excretion (30). The Wilson disease mutation is associated with a failure of Atp7B relocalization and thus restricts copper excretion via the biliary pathway, which facilitates hepatotoxicity. In rat pups, \textit{ATP7B} is not appreciably expressed until late infancy (19), and, thus, copper transport mechanisms responsible for cellular copper excretion, in addition to immature biliary function, may not yet be fully mature in the rhesus monkey at age 1 mo. Thus, maturation in Atp7B localization may also play a role in the different patterns of rhodamine staining we observed in the animals at ages 1 and 5 mo.

Although no effects of copper treatment on indicators of copper status were observed, plasma zinc concentration was significantly lower in animals exposed to high dietary copper. The disappearance of this effect after discontinuation of copper treatment suggests it was a correctable, acute response to high copper intake. An interesting aspect of this study was the assessment of residual long-term consequences of early neonatal copper exposure. Three months after discontinuation of copper treatment (ie, when the animals were 8 mo old), while animals were receiving the recommended amount of dietary copper, retention 22.9%, as compared with 10.2% at age 5 mo. The higher value was still lower than the 64Cu retention measured in the control animals (31.5%), which indicated that 3 mo after discontinuation of the high copper intake, retention was still down-regulated. It is interesting that, at this time, the light-positive copper staining in the liver was no longer detected, and that liver copper concentration diminished further, to 250 \( \mu \text{g/g} \) dry weight. Yet this concentration was still approximately twice that observed in controls. Histologic indicators evaluated by light and electron microscopy were all normal, which suggests that the changes previously detected were reversible.

In summary, in response to a high copper load, infant rhesus monkeys at age 1 mo efficiently down-regulated copper absorption; despite this, there was a remarkable increase in liver copper concentration, which was not associated with histologic evidence of hepatitis, although changes in cell ultrastructure were suggestive of early tissue damage. These changes disappeared 3 mo after discontinuation of copper treatment, which supports that reversibility of the changes. Additional interesting findings include an early increase in the number of Kupffer cells, which may represent a tissue response to excess copper. The lack of intense rhodamine staining suggests that copper was complexed or compartmentalized in a way that was nonreactive to rhodamine. It is well known that neonates have a high liver copper concentration, sometimes even higher than the concentrations observed in patients with Wilson disease, without suffering adverse effects, and our findings support the idea that, during the first months of life, the liver is able to handle considerable amounts of copper without permanent histologic damage.

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MA, BL, SK, and RU wrote the manuscript with the assistance of the other authors. All authors contributed to the design and implementation of the study and to the analysis and discussion of the results, and they approved the final version of the manuscript. None of the authors had any financial or personal conflict of interest.

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S-Equol, a potent ligand for estrogen receptor $\beta$, is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora$^{1-4}$


ABSTRACT

Background: The discovery of equol in human urine more than 2 decades ago and the finding that it is bacterially derived from daidzin, an isoflavone abundant in soy foods, led to the current nutritional interest in soy foods. Equol, unlike the soy isoflavones daidzein or genistein, has a chiral center and therefore can occur as 2 distinct diastereoisomers.

Objective: Because it was unclear which enantiomer was present in humans, our objectives were to characterize the exact structure of equol, to examine whether the S- and R-equol enantiomers are bioavailable, and to ascertain whether the differences in their conformational structure translate to significant differences in affinity for estrogen receptors.

Design: With the use of chiral-phase HPLC and mass spectrometry, equol was isolated from human urine and plasma, and its enantiomeric structure was defined. Human fecal flora were cultured in vitro and incubated with daidzein to ascertain the stereospecificity of the bacterial production of equol. The pharmacokinetics of S- and R-equol were determined in 3 healthy adults after single-bolus oral administration of both enantiomers, and the affinity of each equol enantiomer for estrogen receptors was measured.

Results: Our studies definitively establish S-equol as the exclusive product of human intestinal bacterial synthesis from soy isoflavones and also show that both enantiomers are bioavailable. S-equol has a high affinity for estrogen receptor $\beta$ ($K_i = 0.73$ nmol/L), whereas R-equol is relatively inactive.

Conclusions: Humans have acquired an ability to exclusively synthesize S-equol from the precursor soy isoflavone daidzein, and it is significant that, unlike R-equol, this enantiomer has a relatively high affinity for estrogen receptor $\beta$. Am J Clin Nutr 2005;81:1072–9.

KEY WORDS Equol, soy isoflavones, humans, pharmacokinetics, bacteria

INTRODUCTION

Equol, [7-hydroxy-3-(4'-hydroxyphenyl)-chroman], is a non-steroidal estrogen that was first discovered in the early 1980s in the urine of adults consuming soy foods (1). It was shown to be a key metabolite of daidzin, one of the main isoflavones present in most soy foods, and to be formed after intestinal hydrolysis of the soy isoflavone glycoside (2) and subsequent colonic bacterial biotransformation (3) through an intermediate, dihydroequol (4–6). Equol has an infamous history, having been first identified in the urine of pregnant mares as long ago as 1932 (7) and then in the 1940s having been found to be the environmental estrogenic agent that caused a devastating reproductive disease in sheep, referred to as clover disease (8).

Equol is not of plant origin and is exclusively a product of intestinal bacterial metabolism (9), as evidenced from the finding that infants fed soy formula up to the age of 4 mo (10, 11) and germ-free rats fed soy-containing diets (12) do not make equol. When fed soy protein, a common ingredient of most commercial rodent diets (13, 14), rats and mice are prolific equol producers. In contrast, humans are unique among animals in that, for reasons that remain unclear, only 20–35% of the adult population make equol after ingesting soy foods or being challenged with pure isoflavones (3, 15, 16). Several studies have suggested that those who are equol producers show more favorable responses to soy isoflavone-containing diets (17–21), which leads to the possibility that equol is a more potent isoflavone than genistein (9), which has been so extensively studied in the last decade.

Equol, unlike its precursor daidzein or genistein, is unique in having a chiral carbon atom at position C-3 of the furan ring (Figure 1). It therefore can occur as 2 distinct enantiomeric forms, S-equol and R-equol, which differ significantly in their

1 From the Division of Pathology (KDRS, BEW, LN-Z, and NMB), the Department of Gastroenterology and Nutrition (JEH), and the Department of Pediatrics (SJC and CH), Cincinnati Children’s Hospital Medical Center, and the Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH (KDRS and JEH); the Department of Gastroenterology and Hepatology, University of Perugia, Perugia, Italy (CC and DC); Sanitarium Development and Innovation, Cooranbong, Australia (SJC and CH); the Department of Physiology and Developmental Biology and The Neuroscience Center, Brigham Young University, Provo, UT (EDL); and the Department of Biomedical Sciences, Colorado State University, Fort Collins, CO (TDL and RJH).

2 These findings were presented at the 4th International Symposium on the Role of Soy in Preventing and Treating Chronic Disease, San Diego, CA, November 4–7, 2001, and at the 5th International Symposium on the Role of Soy in Preventing and Treating Chronic Disease, Orlando, FL, September 21-24, 2003.

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4 Address reprint requests to KDR Setchell, Clinical Mass Spectrometry, Cincinnati Children’s Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229. E-mail: kenneth.setchell@cchmc.org.

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conformational structure. We recently showed that S-equol is unique in that it not only possesses estrogenic properties but also is a potent antagonist of dihydrotestosterone in vivo, which has significant implications for the prevention of prostate cancer and other androgen-related conditions (22). We can find no other example of a molecule that is both a selective estrogen and an androgen antagonist. Establishing the diastereoisomerism of equol production is therefore important both in view of the possible differences in biological actions of the enantiomers and in aid of the future development of strategies either to use equol pharmacologically or to manipulate equol production in humans to enhance the effectiveness of soy diets.

When equol was originally isolated, it was found to be optically active (7), and subsequently its enantiomeric assignment as R-equol was questioned and redefined (23–26). The few studies cally active (7), and subsequently its enantiomeric assignment as to enhance the effectiveness of soy diets. pharmacologically or to manipulate equol production in humans

amenable, appropriate weighting schemes (usually 1/

of the enantiomers of equol. For example, it is unclear which form of equol circulates in human blood or is excreted in human urine, because all of the analytic methods for measuring equol in these fluids fail to distinguish the diastereoisomers. To our knowledge, before the current studies, little was known about the pharmacologic or biological activities of the enantiomers. In this report, we focus on several key questions related to equol in humans. First, are the R-, S-, or both enantiomeric forms of equol found in human urine and blood? Second, are intestinal bacteria stereoselective in their synthesis of equol? Third, are both enantiomers absorbed and bioavailable? And fourth, are there differences in the biological activity of the equol’s enantiomers, specifically with regard to their binding affinity for estrogen receptors?

SUBJECTS AND METHODS

Human studies

Identity of the enantiomeric form of equol in human urine and plasma

The characterization of equol’s diastereoisomerism was determined in plasma and urine samples (n = 10 each) selected from study subjects in previous studies (NIH grant no. R01CA73328) of the pharmacokinetics of soy isoflavones in healthy adults consuming soy foods (18). In addition, plasma and urine samples taken from a group of Seventh-Day Adventist vegetarians (n = 10) after they had consumed soymilk (2 × 240 mL/d for 4 d) were analyzed. These samples were collected by staff members of the Pathology Department of the Sydney Adventist Hospital after informed consent was obtained. Characterization of the enantiomeric form of equol was accomplished by chiral-phase HPLC coupled to electrospray ionization–mass spectrometry (ESI-MS) after enzymic hydrolysis with a mixed β-glucuronidase and sulfatase preparation (Helix pomatia), and solid-phase extraction of equol using a Bond Elut C18 cartridge (Varian, Harbor City, CA). The methanolic extracts of urine and plasma were taken for direct analysis by ESI-MS, or, where gas chromatography–mass spectrometry (GC-MS) was used, a volatile tert-butyldimethylsilyl (t-BDMS) ether derivative was prepared as described previously (18, 27, 28).

Written informed consent was obtained from all subjects. The studies were approved by the Cincinnati Children’s Hospital Medical Center Institutional Review Board.

Studies of S- and R-equol bioavailability

Purified samples of S-equol and R-equol (20 mg) obtained by semipreparative chiral-phase HPLC chromatographic separation of a racemic mixture were prepared in capsules and randomly administered orally to 3 healthy adults (1 female and 2 male) on different occasions separated by a washout period of 1 wk. This study was performed by the ethical standards of the Department of Gastroenterology and Hepatology, University of Perugia, Italy, in accordance with the Helsinki Declaration of 1975, as revised in 1983. Each equol enantiomer was taken with a glass of water after an overnight fast and before eating breakfast. Blood samples (10 mL) were obtained via an indwelling catheter placed in the antecubital vein at timed intervals immediately before and then 1, 2, 3, 4, 8, 12, and 24 h after the administration of equol.

These samples were centrifuged for 10 min at 2200 × g and room temperature, and the plasma was removed for the measurement of equol concentrations by stable-isotope dilution GC-MS with selected ion monitoring, as detailed below. The plasma equol concentration–time profiles for the 3 persons were ascertained by using a noncompartmental approach. The WINNONLIN computer program (version 3.0; Pharsight Corporation, Cary, NC) was used for this analysis. The total area under the plasma concentration–time curve (AUC 0→∞; AUCinf) was computed by using the following equation:

\[ \text{AUC}_{\text{inf}} = \text{AUC}(0 \rightarrow t) + C_i/\lambda_z, \]

where t = the last time point for blood sampling (which in this study was 48 h), \( C_i \) = plasma concentration at the last blood-sampling time point, and \( \lambda_z \) = apparent elimination rate constant.

The \( \lambda_z \) was determined from the slope of the best-fitting regression line of the plasma samples in the terminal phase. At least 4 time points were included in the estimation of \( \lambda_z \). When required, appropriate weighting schemes (usually 1/y or 1/y², where y is the observed plasma concentration) were used to improve the goodness of fit. The choice of the number of points included in the terminal phase of the plasma concentration-time curves was based on the weighted residual (difference between model predicted and observed concentrations) values, dispersion of the residual values, and regression coefficient. In all cases, the regression lines were drawn without exclusion of any time points, and the \( R^2 \) values were >0.91. The terminal half-life was calculated as \( \ln(2)/\lambda_z \); the systemic clearance after oral administration was determined as \( \text{dose/AUC}_{\text{inf}} \), and the apparent volume of distribution after oral administration was determined as...
dose/\left(\lambda_{\mu\text{g}}\text{AUC}_{\text{w}}\right)$. Note that “F” used in the abbreviations of these terms (CI/F and Vz/F, respectively) refers to the bioavailable fraction after oral administration, which in this case is unknown.

**Specificity of intestinal bacterial equol production**

Freshly passed stool samples were obtained from 3 recognized equol producers and 3 equol nonproducers for culture of the fecal bacteria exactly as described previously (3). These samples were collected by staff of the Pathology Department of the Sydney Adventist Hospital, Australia, after informed consent was obtained. The freshly passed fecal sample (1 g) was added to 9 mL sterile distilled water and trypticase broth, to which 0.02 mg daidzein was added. The broths were incubated anaerobically for 3 days at 37 °C. A 2-mL aliquot of this incubation mixture was taken, diluted with distilled water (2 mL), and centrifuged for 5 min at 2200 \(\times\) g and at room temperature. The supernatant was passed through a Bond Elut C18 solid-phase cartridge to extract the isoflavones, which were recovered by elution with methanol (5 mL). This sample was subjected to chiral-phase HPLC coupled with ESI-MS to separate and identify the S- and R-equol diastereoisomers as described below. Confirmation of the identity of each enantiomeric form of equol was based on its chromatographic retention time and mass spectrum as compared with pure standards of S- and R-equol.

**Animal study—identification of S-equol in rat urine**

A sample of adult Sprague-Dawley rat urine was obtained from animals used in a previously published study of rodents fed commercial chow containing soy isoflavones that was performed with the approval of the Cincinnati Children’s Hospital Medical Center Animal Use Committee (14).

**Analytic methods**

Equol concentrations were measured in the urine and plasma by either ESI liquid chromatography–MS (ESI-LC-MS) or GC-MS techniques, or both, after liquid-solid extraction, hydrolysis of the conjugates with a mixed \(\beta\)-glucuronidase and sulfatase enzyme preparation (Helix pomatia; Sigma Chemicals, St Louis, MO), reextraction, and preparation of a volatile derivative in the case of GC-MS analysis. The methods have been outlined in previous studies of isoflavones (2, 18, 27, 28). Separate quantification of the S- and R-equol was achieved by using a recently developed chiral-phase HPLC chromatographic method (described below) that resolves the S- and R-enantiomers of equol.

**Determination of S- and R-equol concentrations in plasma by gas chromatography–mass spectrometry**

The concentrations S- and R-equol in plasma (0.5 mL) samples collected after administration of these enantiomers were measured by GC-MS after the addition of \(\left[13\text{C}\right]\) stable isotope-labeled analogs of S- and R-equol as internal standards for quantification. These internal standards were obtained by chiral-phase HPLC chromatographic separation of \(\left(\pm\right)\left[13\text{C}\right]\)equol that was synthesized from \(2,3\text{-}13\text{C}\)daidzein as described else where (30). Isoflavones were extracted on a solid-phase octadecylsilane-bonded silica cartridge and hydrolyzed enzymatically with a mixed \(\beta\)-glucuronidase and sulfatase preparation (H. pomatia). After reextraction and purification, the t-BDMS ether derivatives were prepared, and the derivatized samples were analyzed by selected ion monitoring GC-MS as described previously (18, 27, 28).

**Chiral-phase HPLC and electrospray ionization–mass spectrometry separation and identification of diastereoisomers of equol**

Sample extracts for the characterization of the enantiomeric form of equol in urine and plasma were taken up in 100 \(\mu\)L of the HPLC mobile phase, and a 20-\(\mu\)L sample was injected on column. Separation of the enantiomers of equol was performed on a Chiralcel column (Chiral Technologie, Inc, Exton, PA). The mobile phase used to enable baseline resolution of S- and R-equol was a gradient elution beginning with 90% hexane and 10% ethanol (by vol) and linearly increasing to a final composition of 10% hexane and 90% ethanol (by vol) over a 15-min period at a flow rate of 1 mL/min. Detection of S- and R- equol is possible by ultraviolet absorption at 260 nm, even though equol has poor ultraviolet absorption characteristics, provided relatively high concentrations are injected on column. This wavelength was monitored to establish the conditions using the pure standards of equol. However, for detecting equol enantiomers in human plasma and urine, the greater sensitivity of ESI-MS was necessary. ESI-MS was performed on a Micromass Quattro LC/MS (Waters Corp, Milford, MA). The HPLC effluent to the ESI probe was split 10:1. The desolvation temperature was 300 °C, and the source temperature was 100 °C. The sampling cone was held at 50 V and the extractor at 2 V. Data were collected in the negative ion mode, and the [M-\(\text{H}^-\)] ions monitored were a mass-to-charge ratio (m/z) of 241 (equol) and a m/z of 242 (2,13\text{C})equol. The identity of the enantiomeric form of equol in the human plasma and urine samples was based on the retention time of the eluting peak in the mass chromatogram compared with the mass chromatograms obtained for pure standards of S- and R-equol analyzed under identical conditions.

**Studies of estrogen receptor binding**

**Synthesis of hormone receptor proteins**

Full-length human estrogen receptor (ER) \(\alpha\) [\(\text{ER}\alpha\) pcDNA-ER\(\alpha\); RH Price, University of California, San Francisco] and rat ER\(\beta\) [pcDNA-ER\(\beta\); TA Brown, Pfizer, Groton, CT] expression vectors were used to synthesize hormone receptors in vitro by using the transcription and translation–coupled reticulocyte lysate system (Promega, Madison, WI) with T7-RNA polymerase, during a 90-min reaction at 30 °C. Translation reaction mixtures were stored at −80 °C until further use.

**Saturation isotherms**

To calculate and establish the binding affinity of the R and S equol enantiomers for ER\(\alpha\) and ER\(\beta\), 100-\(\mu\)L aliquots of reticulocyte lysate supernatant were incubated at optimal time and temperature—90 min at room temperature (ER\(\beta\)) and 18 h at 4 °C (ER\(\alpha\))—with increasing (0.01–100 nmol/L) concentrations of \([\text{H}^1]17\beta\)-estradiol (E\(\text{Z}\)). These times were determined empirically, and they represent optimal binding of receptor with estrogen. Nonspecific binding was assessed by using a 300-fold excess of the ER agonist diethylstilbestrol in parallel tubes. After incubation, bound and unbound \([\text{H}^1]E\text{Z}\) was separated by passing the incubation reaction through a 1-mL lipophilic Sephadex LH-20 column (Sigma-Aldrich Co, St Louis, MO). Columns
were constructed by packing a disposable pipette tip (1 mL; Labcraft, Curtin Matheson Scientific, Inc, Houston, TX) with TEGMD (10 mmol Tris-Cl/L, 1.5 mmol EDTA/L, 10% glycerol, 25 mmol molybdate/L, and 1 mmol dithiothreitol/L; pH 7.4)-saturated Sephadex according to previously published protocols (31, 32). For chromatography, the columns were reequilibrated with TEGMD (100 μL), and the incubation reactions were added individually to each column and allowed to incubate on the column for an additional 30 min. After this incubation, 600 μL TEGMD was added to each column, flow-through was collected, 4 mL of scintillation fluid was added, and samples were counted (5 min each) in a 2900 TR Packard scintillation counter (Packard Bioscience, Meriden, CT).

RESULTS

Characterization of S-equol in humans and rats by chiral-phase HPLC and mass spectrometry

A typical separation of S- and R-equol enantiomers when a racemic mixture was subjected to chiral-phase chromatography and the isoflavones were detected by their ultraviolet absorbance at 260 nm is shown in Figure 2. S-equol eluted from the chiral-phase column with a shorter retention time than that of R-equol, and baseline resolution was achieved. The identity of both enantiomers was confirmed by isolation of both peaks and measurement of optical dichroism. Also shown in Figure 2 is a typical HPLC-ESI-MS mass chromatogram of the negative ion m/z 241 corresponding to the pseudomolecular ion ([M–H]⁻) of equol obtained from the analysis of hydrolyzed extracts of human urine. All samples of human urine analyzed were found to contain one major peak in all samples analyzed, and this had a retention time corresponding to that of the S-equol standard.

FIGURE 2. Chiral-phase HPLC separation with ultraviolet detection (260 nm) showing resolution of a standard mixture of S- and R-equol (left). These profiles are compared with the superimposed ESI-MS mass chromatograms of mass-to-charge ratio (m/z) 241 ([M–H]⁻) ion obtained from the analysis of hydrolyzed extracts of human and rat urine collected after ingestion of soy foods (right), which established S-equol as the only enantiomer excreted in human urine. ESI-MS, electrospray ionization–mass spectrometry.

Pharmacokinetics of S- and R-equol enantiomers

Mean (±SEM) appearance and disappearance concentration curves for equol in plasma after single-bolus oral administration of R-equol and S-equol to 3 healthy adults are shown in Figure 3. The administration of both enantiomers yielded similar plasma pharmacokinetic profiles, which confirmed that the 2 diastereoisomers are similarly bioavailable. Equol rapidly appeared in plasma and disappeared with a terminal elimination half-life of 4.9 ± 1.6 and 6.2 ± 0.2 h, respectively, for the S- and R-enantiomers, and there was no obvious difference between these values. A comparison of the computed pharmacokinetics of the diastereoisomers is shown in Table 1. There were no statistical differences in maximum plasma concentration, time to reach maximum plasma concentration, terminal elimination half-life, AUC_Linf apparent volume of distribution, and systemic clearance between R- and S-equol, and no difference in the absorption rates of the 2 enantiomers.

Because GC-MS was used to quantify equol in these plasma samples, and thus this technique cannot resolve the individual enantiomers as their α-BDMS ether derivatives, it was essential to select a number of samples (n = 10) of human plasma collected from equol producers who had consumed 2 × 240 mL soymilk. Consistent with the urinary analysis, the ESI-MS profiles of plasma showed one major peak in all samples analyzed, and this had a retention time corresponding to that of the S-equol standard.

TABLE 1

Computed plasma pharmacokinetics for S- and R-equol administered to 3 healthy adults in a single-bolus oral dose of 20 mg of each diastereoisomer in a randomized crossover design with a 1-wk washout interval

- All values are ± SD. T_max time to reach maximum plasma concentration; C_{max} maximum plasma concentration; t_{1/2}, terminal elimination half-life; Vz/F, apparent volume of distribution; Cl/F, systemic clearance; AUC_{Linf} total area under the plasma concentration-time curve. There were no significant differences between S- and R-equol.
Evidence for enantiomer-specific synthesis of S-equol by human intestinal bacteria

In vitro studies were performed on cultured human fecal flora collected from healthy adults who were challenged for 4 d with soy foods and who were determined from plasma and urinary isoflavone analysis to be either equol producers or equol non-producers as defined previously (9). Daidzein (20 mg), the precursor isoflavone of equol, was then incubated with cultured fecal flora at 37 °C for 72 h; after extraction of the supernatant fluid by solid-phase chromatography, the extract was analyzed by direct ESI-MS with chiral-phase HPLC separation. Superimposed mass chromatograms of the negative ion recordings for m/z 241 ([M-H]⁻ ion) that were specific for a pure standard of S-equol and for the equol isolated from the 72-h supernatant fluid from one of the equol producers and from one equol nonproducer are shown in Figure 5. The cultured fecal flora from the equol producers showed a peak corresponding to equol that had a retention time identical to that of the authentic pure standard of S-equol. By contrast, the supernatant fluid from an equol non-producer showed negligible production of S-equol. These results establish conclusively that fecal bacteria are selective in producing only S-equol as the principal metabolite of the soy isoflavone, daidzein.

Estrogenic activity of equol enantiomers

Competitive binding studies were used to assess the estrogenic properties of R- and S-equol. On the basis of the ability of R- and S-equol to compete with [³H]E₂ in ER binding, their affinities for ERs translated in vitro were shown to be very different. S-equol showed the greatest affinity for ERβ (Kᵦ = 0.73 ± 0.2 nmol/L), whereas its affinity for ERα (Kᵦ = 6.41 ± 1 nmol/L) was relatively poor. In contrast, R-equol possessed only 4.8% and 25.0% as much relative binding affinity, respectively, for ERβ (Kᵦ = 15.4 ± 1.3 nmol/L) and for ERα (Kᵦ = 27.38 ± 3.8 nmol/L) as did S-equol. For comparison, 17β-estradiol binds ERα with a Kᵦ of 0.13 nmol and ERβ with a Kᵦ of 0.15 nmol. S-equol thus shows ER selectivity with a high affinity for ERβ, whereas R-equol can, at best, be classified as a weak estrogen.

DISCUSSION

In 1932, Marrian and Haslewood, working at the University College London, first isolated and elucidated the chemical structure of the isoflavone metabolite equol (7). It was found as a minor metabolite of the urine of pregnant mares and shown to be optically active, although in subsequent years there was some confusion as to its enantiomeric assignment; it was first assigned the R-configuration, and only later was the absolute configuration defined as the S-enantiomer (25). The importance of defining the nature of the stereoisomerism in humans relates to the marked differences in the conformational structure of the diastereoisomers and the expectation that there would be differences in the biological activity, primarily related to binding to the ER. When equol was first isolated, it was reported by Marrian and Haslewood to have no estrus-producing activity when injected into ovariectomized mice in doses as large as 0.86 mg/animal (7). This observation was inconsistent with the later finding that
equol was the estrogenic agent responsible for the devastating reproductive abnormalities, referred to as clover disease, observed in sheep in the mid-1940’s (8, 33, 34). Later, in a period that predated knowledge of specific ER subtypes (35, 36), equol was shown in vitro to bind to uterine cytosolic ERs (37, 38). Given the predominance of ERs in the uterus, it is almost certain that these early reports of binding affinities refer to equol’s binding to ERα, rather than to ERβ. Using preparations of recombinant steroid receptors, we have shown that only the S enantiomer of equol binds to ERs with sufficient affinity to be of physiologic relevance based on circulating equol concentrations typically found in humans (9). Furthermore, almost 50% of equol circulates unbound to serum protein (39), whereas endogenous estrogen is >95% protein bound. This protein-binding status of equol is likely to enhance its biological potency because it is only the “free” or unbound fraction that is available for receptor occupancy. The relative binding affinity of the R- and S-equol enantiomers for ERα were 0.47% and 2.0% with that of 17β-estradiol. However, S-equol is largely ERβ selective and has a relatively high affinity for this receptor subtype. S-equol binds ERβ ~20% with as much affinity as does 17β-estradiol (equol: Kᵢₛ = 0.7 nmol/L; 17β-estradiol: Kᵢₛ = 0.15 nmol/L), whereas the R enantiomer bound at ~1% of the affinity. These findings are corroborated by several studies (40–42) that also show equol to have selective affinity for ERβ, and, therefore, equol can be defined as a type of selective ER modulator.

As a potent antagonist of dihydrotestosterone (DHT) in vivo, equol is also unique, in that we can find no other example of a compound that has selective estrogen action and yet also has the ability to be an antagonist of androgen action (22). It is interesting that the mechanism of its anti-androgen action differs from that of the anti-androgen drugs used in clinical practice to block the effects of DHT. For example, equol has no affinity for the androgen receptor (22) and therefore does not function as an androgen receptor blocker. It also does not appear to alter the synthesis of DHT in the way that 5α-reductase inhibitors do, but, rather, it appears to bind directly to DHT (22), and this effect is seen with both R- and S-equol (TD Lund, RJ Handa, ED Lephart, KDR Setchell, unpublished data, 2003).

Given the distinctly different biological actions of the diasteroisomers of equol, particularly with regard to their affinity toward ERs, it is relevant to define the stereoisomerism of equol production in humans. Using ESI-MS with selected ion monitoring, we analyzed urine and plasma samples from healthy adults, and by our ability to separate the 2 diastereoisomers with the use of chiral-phase column chromatography, we have shown for the first time that S-equol is the only enantiomer circulating in human blood and excreted in urine (Figures 2 and 3). This is also true in the rat, a species that is predominantly an equol producer (14). The logical explanation for the finding of a single enantiomer in plasma and urine is that intestinal bacteria are stereoselective in their synthesis, but the possibility that both enantiomers would be made in the intestine, but only one, the S enantiomer, would be absorbed required addressing. Furthermore, racemization of R-equol to S-equol during the former’s absorption was an alternative possibility that was feasible and required investigation.

The separate oral administration of pure S- and R-equol to 3 healthy adults clearly showed that both enantiomers, when present in the intestine, are efficiently absorbed and appear rapidly in plasma. There were no differences in the pharmacokinetics of the 2 enantiomers. The bioavailability of equol as measured by the dose-adjusted AUCₘₙ is relatively high when compared with the bioavailability of genistein and daidzein reported in previous studies (18, 27, 28). The clearance rate of equol was also much slower than that of the soy isoflavones, which contributes to the maintenance of high circulating equol concentrations observed in rodents (14). ESI-MS established that, after its administration, R-equol appeared in plasma unchanged, and therefore the possibility of bacterial production of R-equol in the intestine with racemization to S-equol during absorption can be confidently excluded. Thus, these data taken together are indicative of the enantiomer-specific production of S-equol by intestinal bacteria. This is now confirmed by in vitro experiments in which human fecal flora from equol producers were cultured under anerobic conditions and incubated with daidzein or soy isoflavones. After 72 h in culture, S-equol was the sole enantiomer identified in the supernatant. Thus, given that humans, rats, and sheep all produce S-equol—and it is likely that macaque monkeys (43), chimpanzees (44), dogs (45), domestic fowl (46), cows (47), and mice (14) also do so—it is evident that the bacteria responsible for equol production are all highly selective in performing an asymmetric synthesis with production of the one enantiomer that shows the highest ligand affinity for ERβ.

The formation of equol from its precursor daidzein proceeds through an intermediate, dihydrodaidzein. Our pharmacokinetic studies show that equol is rapidly absorbed from the intestine, but its formation after the initial intake of daidzein or of soy foods containing daidzein or daiztein is a time-dependent process. It generally takes >12 h for equol to appear in the plasma, and, in some adults, it may not appear for 36 h, which indicates that the colon is the site of its formation (18, 28, 48). Identification of the bacteria responsible for equol production has thus far been elusive. It is apparent that there is more than one bacterium involved because we have observed cases in which dihydrodaidzein is present in urine in the absence of equol, which is consistent with partial conversion of daidzein to equol (KDR Setchell, unpublished observations, 1995). Furthermore, in vitro incubation of fecal homogenates from some adults was shown to produce dihydrodaidzein and O-desmethylangolensin but not equol, whereas recently it was shown that some antibiotics, such as rifampicin and kanamycin, may inhibit the production of equol but not of dihydrodaidzein (6). In contrast, kanamycin virtually eliminated equol production in the plasma of cynomologus monkeys (49), which highlights the complexity of the bacterial production of equol. Attempts to identify the species of bacteria involved in equol production have yielded some information regarding strains that are capable of hydrolyzing the β-glucoside of daidzein (50, 51) or of converting daidzein to dihydrodaidzein (52), and one report claimed that Streptococcus intermedius spp, Ruminococcus productus spp, and Bacteroides ovatus in vitro perform the required conversion (53).

In view of the apparent advantages of being an equol producer (9, 17, 19–21, 54), the question of whether it is possible to manipulate the intestinal milieu in favor of equol production when soy foods are given is relevant. Early studies by Setchell and Cassidy (55) using an in vitro model of human colonic fermentation showed that, with a background of a high nonstarch polysaccharide environment, which affords increased colonic fermentation, the conversion of daidzein to equol is complete, but
REFERENCES


β-Carotene–rich orange-fleshed sweet potato improves the vitamin A status of primary school children assessed with the modified-relative-dose-response test\textsuperscript{1–3}

Paul J van Jaarsveld, Mieke Faber, Sherry A Tanumihardjo, Penelope Nestel, Carl J Lombard, and Ambrose J Spinnler Benáde

ABSTRACT
Background: β-Carotene–rich orange-fleshed sweet potato (OFSP) is an excellent source of provitamin A. In many developing countries, sweet potato is a secondary staple food and may play a role in controlling vitamin A deficiency.

Objective: The objective was to determine the efficacy of daily consumption of boiled and mashed OFSP in improving the vitamin A status of primary school children.

Design: Children aged 5–10 y were randomly assigned to 2 groups. The treatment group (n = 90) consumed 125 g boiled and mashed OFSP (1031 retinol activity equivalents/d as β-carotene), and the control group (n = 90) consumed an equal amount of white-fleshed sweet potato devoid of β-carotene for 53 school days. All children were dewormed to exclude helminthic infection. The modified-relative-dose-response test for vitamin A status was conducted before and after intervention.

Results: The estimated intervention effect for the ratio of 3,4-didehydroretinol to retinol (DR:R) was \(0.008\) (95% CI: \(-0.015, -0.001\); \(P = 0.0203\)), which indicated a greater improvement in vitamin A liver stores in the treatment group than in the control group. The proportions of children with normal vitamin A status (DR:R < 0.060) in the treatment group tended to increase from 78% to 87% (\(P = 0.008\)) and did not change significantly (from 86% to 82%) in the control group (\(P = 0.267\)). These proportions were not used to test the intervention effect or within-group changes because the study was powered to test the intervention effect on DR:R.


KEY WORDS Vitamin A status, modified relative dose response, β-carotene, orange-fleshed sweet potato, efficacy, school-feeding program, South Africa

INTRODUCTION
Vitamin A deficiency is of public health significance in the developing world. Globally, 140 million children aged <5 y, of whom nearly 100 million live in South Asia or sub-Saharan Africa, have low serum retinol concentrations (<0.7 μmol/L) (1). Countries of eastern and southern Africa have the highest prevalence (37%) of preschool children with low serum retinol concentrations, followed by South Asia (35%) and Western and Central Africa (33%) (1). In South Africa, 1 in 3 preschool children has a serum retinol concentration <0.7 μmol/L (2), and 55–68% of children aged 1–9 y consume <50% of the recommended dietary intake of vitamin A (700 μg retinol equivalents) (3); children living in rural areas are the most affected (2, 3).

Vitamin A deficiency is caused by a habitual diet that provides too little bioavailable vitamin A to meet physiologic needs. Rapid growth and frequent infections, which cause ineffective utilization of the vitamin, are also critical factors (4). The dietary sources of vitamin A are preformed vitamin A (commonly found in foods of animal origin) and provitamin A carotenoids (found in yellow and orange-fleshed fruit and vegetables and in dark-green leafy vegetables). Of the \(≈600\) carotenoids found in nature, only 3 are important precursors of vitamin A in humans, namely, β-carotene, α-carotene, and β-cryptoxanthin. β-Carotene is the major provitamin A component of most carotenoid-containing foods (5).

Strategies to control vitamin A deficiency include dietary diversification, food fortification, and vitamin A supplementation. Dietary diversification includes the production of β-carotene–rich crops, such as orange-fleshed sweet potato (OFSP). The consumption of OFSP increased the vitamin A intake of Kenyan women and children (6). A recent ex ante impact assessment indicated that OFSP could make a major contribution to controlling vitamin A malnutrition in sub-Saharan Africa (7). Replacing white-fleshed varieties with high β-carotene varieties that meet \textsuperscript{1}From the Nutritional Intervention Research Unit, Medical Research Council, Parow, South Africa (PJvJ, MF, and AJSB); the Biostatistics Unit, Medical Research Council, Parow, South Africa (CIL); Harvest Plus, IFPRI, Washington, DC (PN); University of Wisconsin, College of Agricultural and Life Sciences, Department of Nutritional Sciences, Madison, WI (SAT).

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\textsuperscript{3}Reprints not available. Address correspondence to PJ van Jaarsveld, Nutritional Intervention Research Unit, Medical Research Council, PO Box 19070, Tygerberg 7505, South Africa. E-mail: paul.van.jaarsveld@mrc.ac.za.

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local preferences could benefit an estimated 50 million children aged <6 y who are currently at risk of diseases associated with vitamin A deficiency. The consumption of diets containing mostly plant sources of \( \beta \)-carotene, the primary source being red sweet potato, increased serum retinol concentrations in Indonesian children marginally deficient in vitamin A (8).

Under the International Potato Center’s Vitamin A for Africa partnership, \( \beta \)-carotene–rich OFSP varieties are widely promoted. It is important to determine the efficacy of \( \beta \)-carotene–rich OFSP in improving vitamin A status in children. Because of its agronomic, technologic, and acceptability performance, the naturally high \( \beta \)-carotene–containing Resisto variety of sweet potato, which has a dark orange root, was chosen for the present study. This variety was originally developed in the United States through conventional breeding and was successfully introduced into a home-gardening project in South Africa (9) after being judged by members of the community to be tastier than the other varieties tested.

This study determined the efficacy of the daily consumption of boiled and mashed \( \beta \)-carotene–rich OFSP in improving vitamin A status, measured by the modified-relative-dose-response (MRDR) test, in primary school children participating in a school feeding program.

SUBJECTS AND METHODS

Study population

The study was conducted from March (end of the summer season) to June (during the winter season) 2002. The study subjects were 5–10-y-old Grades 1–3 primary school children from an area =40 km northwest of Durban, KwaZulu-Natal Province, and 26 km from an area with a known high prevalence of vitamin A deficiency (10) and a low dietary vitamin A intake (11). Most of the residents in the study area were of low socioeconomic status. Children received a cooked meal 4 d/wk, excluding Mondays, through a school feeding program for the first 3 wk of intervention. Thereafter, cooked meals were provided on Mondays until the end of the study. Meals were prepared by a member of the community and consisted primarily of rice with cabbage and potatoes or beans and, occasionally, samp (crushed maize kernels) with beans. The meals always contained sunflower seed oil, fried onions, and salt. Flavorings were also used and included curry powder, beef stock cubes, or soya mince (soybean protein with added spices and herbs). The community in which the study was conducted was unfamiliar with OFSP varieties; however, they were familiar with white-fleshed sweet potato (WFSP) varieties.

The Ethics Committee of the South African Medical Research Council approved the study. Permission and support for conducting the study was obtained from the school governing body, the school principal, and the educators. The purpose, procedures, nature, and potential benefits of the study were explained to the parents or legal guardians of children in Grades 1–3. Those parents who agreed that their children could participate in the study signed an informed consent form.

Study design

The study was a randomized controlled unmasked feeding trial. All biochemical measurements were, however, masked. Vitamin A status was measured using the MRDR test, in which the ratio of serum 3,4-didehydroretinol (DR) to retinol (R), DR:R (mol/mol), is calculated (12). Sample size calculations indicated that 86 children per group were needed to detect a 0.02 difference in serum DR:R between groups, assuming an SD of 0.04 in each group at a 5% significance level and 90% power. An additional 5% of children were included to compensate for potential loss to follow-up.

Children were excluded from the study if their parent or legal guardian did not sign the informed consent form, the child was unwilling to participate, or the hemoglobin concentration was <90 g/L. Eligible children were stratified by classroom and sex and were randomly assigned to 1 of the 2 groups within blocks of 8 children listed alphabetically. One group (\( n = 90 \); treatment group) received boiled and mashed \( \beta \)-carotene–rich OFSP [Resisto variety; 1031 retinol activity equivalents (RAE)/d] and the other (\( n = 90 \); control group) an equal amount of boiled and mashed WFSP (Bosbok variety; 0 RAE/d).

All the children in Grades 1–3 received one 500 mg mebendazole chewable anthelmintic tablet to exclude helminthic infection as a confounder. Treatment was given 1 mo before the baseline survey, 1 wk before the baseline survey, and 1 mo before the end measurements were taken.

Sweet potato cultivation and supply

To ensure that sweet potatoes of high quality were available, and to control and closely monitor the cultivation program, both the OFSP and WFSP were cultivated at the Vegetable and Ornamental Plant Institute of the Agricultural Research Council, Roodeplaat, Gauteng Province.

Cultivation was staggered so that specified amounts of sweet potatoes of similar age and maturity, especially with regard to the \( \beta \)-carotene content of the OFSP variety, could be harvested on predetermined dates. A 10-d supply of sweet potatoes was harvested biweekly, packed in 10-kg color-coded mesh bags, and transported 600 km overnight to the school. Once at the school, the sweet potatoes were kept at room temperature in a lockable room.

Intervention

Children were fed sweet potato 5 d/wk during the 1030 mid-morning break. The usual school meal was served after the sweet potato serving was consumed. No intervention took place, ie, no sweet potato was fed, during the 8-d April school holiday, weekends, and public holidays. Sweet potato was provided to the children for 53 d over 10.6 wk.

Each child was issued with a color-coded identification label that showed the child’s name, code, and school grade. Feeding bowls, files, sheets to record compliance, and kitchenware were also color-coded. Each classroom had a monitor recruited from the local community and trained to monitor and record daily compliance and the reasons for absence from school. Compliance was defined as the number of days that a child received and ate all of the sweet potato expressed as a percentage of the total number of days that sweet potato was provided.

Preparing and feeding the sweet potato

The sweet potatoes were cooked in the school kitchen, which was also used for preparing the school meal. Two cooks, one for cooking the OFSP and the other for cooking the WFSP, were recruited from the local community and trained to follow the
standardized procedure for preparing the sweet potato; ≈15 kg OFSP and 22 kg WFSP were cooked on each school day. The extra WFSP was fed to all nonparticipating children in each classroom.

Every school day, usually between 0740 and 0840, the sweet potatoes were washed and cooked unpeeled; small-sized sweet potatoes were kept intact, and medium to large-sized sweet potatoes were cut in half. The OFSP and WFSP were cooked separately in large pots. In general, only one-half of the depth of the sweet potatoes was covered with water and boiled in a closed pot until soft (≈55 min). The water was drained off before the sweet potatoes were peeled and mashed by hand. To promote the absorption of β-carotene, and given that oil was included in the school meal, sunflower seed oil was added to the mashed sweet potato: 140 mL to the OFSP and 200 mL to the WFSP, equivalent to 1.0–1.4 mL oil per portion of sweet potato served, depending on the amount of sweet potato left after peeling. After the oil was thoroughly mixed with the mashed sweet potato, the sweet potato was transferred to color-coded containers and taken to the classrooms. Classroom monitors observed the cooks serving the sweet potatoes. Ice cream scoops were used, which provided typical portion sizes of 125 g.

During feeding, the treatment and control groups were seated on opposite sides of the classroom to avoid the exchange or sharing of sweet potato. Consumption took place under close supervision by the classroom monitors, and the children were encouraged, but not forced, to eat the whole portion. A short questionnaire on the acceptability of the OFSP was completed for the treatment group at the end of the study.

Sampling and analysis of boiled and mashed sweet potato for β-carotene content

Samples of boiled and mashed OFSP and WFSP were collected 5 times during the intervention. Duplicate portions of ≈150 g each were collected from 2 different parts of the cooking pot on 3 consecutive days each time. Samples were put into plastic freezer bags and frozen within 3 h to −20 °C. To limit the degradation of β-carotene by oxygen during storage, air was squeezed from the sample bag before it was sealed. Once the 3-d sample collection was completed, the frozen samples were transported in a cool box containing ice packs to the laboratory of the Nutritional Intervention Research Unit, Cape Town, and stored at −80 °C until analyzed.

To determine the average sweet potato serving (in g) and the amount of β-carotene consumed, the weight of 33% of all servings was measured (n = 30 per group), on the same days that the sweet potato samples were collected, with an electronic digital computing scale (model MW-1200, Casbee Massicot; CAS Corp, Gangdong-Gu, Seoul, Republic of Korea).

The β-carotene content of the samples was determined within 3 mo after the last 3-d collection period. The 3 duplicate samples per collection period were combined, and 5 composite samples were analyzed in triplicate by HPLC (SpectraSERIES; Thermo Separation Products, Fremont, CA) on the same day by using a validated method established for this study. Three 2-g samples of each composite sample were extracted with tetrahydrofuran: methanol (1:1, by vol). A β-carotene standard (synthetic, crystalline, Type II, product C-4582; Sigma Chemical Co, St Louis, MO) was purified by HPLC, and an aliquot of the purified standard solution with a known concentration was used as the external standard for quantification of β-carotene in the sample extract.

Anthropometric measures

Anthropometric measurements were taken at baseline. Children were weighed while they were wearing light clothing and no shoes to the nearest 0.05 kg on a load-cell-operated digital scale (UC-300 Precision Health Scale; A&D Co Ltd, Tokyo, Japan). Children were measured without shoes to the nearest 0.1 cm with a vertically placed wooden board fitted with a measuring tape and a movable headpiece.

Assessment of vitamin A status

The MRDR test was used to determine the adequacy of liver vitamin A stores (12, 13). Between 0845 and 0900, 45–48 children per day were given a single oral dose of 2 mg (7.0 μmol) 3,4-didehydroretinyl acetate in 190 μL corn oil. To promote its absorption, each child was given a high-fat, low vitamin A-containing snack (30 g peanut butter on a 30-g slice of white bread) and a 250-mL cold drink (concentrated nonnutritive orange-flavored drink diluted with water) that was consumed under supervision.

During the baseline and follow-up surveys, the school meal was standardized (cabbage and rice) and devoid of vitamin A to exclude the effect that different meal compositions might have on the MRDR test. For the same reason, a 10-d washout period during which sweet potato was not served was implemented before the final blood samples were obtained.

The serum concentration of DR reaches its maximum ≈5 h after dosing in vitamin A–depleted children. Blood (6 mL that was also used for the biochemical measurements) was drawn from each child as close as possible to the child’s 5 h ± 15 min time point after 3,4-didehydroretinyl acetate had been administered. Blood samples were taken between 1350 and 1430 h in the same sequence as the dosing. DR:R, or an MRDR value ≥0.060, indicated inadequate vitamin A liver stores.

Biochemical measurements

To ascertain the baseline nutritional status of participating children, iron and zinc status were determined. Blood was obtained by antecubital venipuncture in nonfasting subjects. Immediately after collection, 1.0 mL of the sample was removed to determine the hemoglobin concentration by the cyanmethemoglobin method with a portable photometer (Product no. 7316; Ames Minilab, Miles Inc, Elkhart, IN). A blood sample of known hemoglobin concentration was used as the reference. The remaining blood was transferred to a sterile Gel and Clot Activator tube (SST II Plus; Becton Dickinson and Co, Vacutainer Systems, Plymouth, United Kingdom). The latter was centrifuged (750 × g for 10 min at room temperature), and aliquots of serum were transferred to a series of containers that were put in a cool box containing ice packs. The serum was frozen later the same afternoon at −20 °C. All field procedures were done as quickly as possible, and care was taken throughout to protect the blood and serum samples from direct light. All tubes were trace element–free to avoid contamination with zinc and iron.

After completion of each of the baseline and follow-up surveys, which took 4 d each, the serum samples were transported to
the Nutritional Intervention Research Unit, Cape Town, and stored for 1–6 mo at −80 °C until analyzed. Serum concentrations of retinol and DR were measured in the same injected sample (13) by reversed-phase HPLC (SpectraSERIES). The baseline and postintervention serum of a subject were analyzed on the same day to minimize intrasay variation in retinol and DR concentrations. Serum ferritin was determined with an immunoassay in medium (Ferritin MAB Solid Phase Component System; ICN Pharmaceuticals, Orangeburg, NY) with the use of an external control sample (Ligand 1, 2, 3, Chiron Diagnostics Ltd, Halstead, United Kingdom). Serum zinc was analyzed with a flame atomic absorption spectrophotometer (Philips Pye Unicam SP9, Cambridge, United Kingdom) (14) with a commercial control serum (Seronorm Trace Elements Serum; SERO AS, Billingsstad, Norway) as a quality control. Because hemoglobin, ferritin, retinol binding protein, and zinc are acute phase reactants, C-reactive protein (CRP) and α1-acid glycoprotein (AGP) were measured as markers of infection. CRP was measured with an immunoturbidimetric method (Technicon method no. SM4-0183G89, Technicon RA-1000 auto-analyzer; Technicon Instruments, NY) with the use of Bayer TESTpoint Serum Protein Controls (Bayer Diagnostics, Fernwald, Germany). AGP was measured with an immunoturbidimetric technique and with nephelometry with an in vitro diagnostic reagent kit (Dade Behring, Marburg, Germany).

**Statistical analysis**

Data were entered in Microsoft EXCEL (Redmond, WA) and analyzed with the use of SPSS 10.0 for WINDOWS (SPSS Inc, Chicago, IL). Baseline means ± SDs for the biochemical measurements and anthropometric indexes were obtained for the treatment and control groups. Baseline differences for the anthropometric and biochemical measurements between groups were examined to verify the success of the randomization using t tests and chi-square tests. Data were analyzed on an intent-to-treat basis. Individual-level changes (postintervention value minus baseline value) were calculated. Tests for normality of the values were not rejected, and the appropriate value for significance (P value) was applied. The test for paired data was used to compare pre- and postintervention values within each group. P values < 0.05 were considered statistically significant. The proportions of children with normal liver stores (DR:R < 0.060) were not used to test the intervention effect and within-group changes because the study was powered to test the intervention effect on DR:R. The reported proportions of children with normal liver stores are only explanatory.

An analysis of covariance (ANCOVA) was carried out to investigate the effect of the number of days a child was absent from school (and hence did not receive sweet potato) after adjustment for the baseline value of the postintervention biochemical measurement. The number of days absent was not a significant factor in the ANCOVA model, and the estimated intervention effects concurred with the pre-post analysis reported in the text.

Height-for-age z scores (HAZ), weight-for-age z scores (WAZ), and weight-for-height z scores (WHZ) were obtained following World Health Organization procedures (15). The birth date of each child was obtained from the school register. Anemia was defined as a hemoglobin concentration < 115 g/L (16) and zinc deficiency as a serum zinc concentration < 10.7 μmol/L (17). The acute phase response was defined as serum concentrations of CRP > 10 mg/L and AGP > 1.2 g/L (18). Three subsets of data were further examined with the following exclusions: 1) only CRP was elevated, 2) only AGP was elevated, and 3) both CRP and AGP were elevated. Because the estimated intervention effects for DR:R, the DR dose response, and serum retinol in the above 3 subanalyses were not different from the estimates of the complete data set, only the latter are reported.

**RESULTS**

Two children had a hemoglobin concentration < 90 g/L and were excluded from the study and referred to the local clinic for treatment. Of the 180 children enrolled, 178 completed the study; one child in each group was absent when blood was drawn at the end of the study. The birth date was not available for one child in the intervention group and for 3 children in the control group; thus, their anthropometric indexes could not be calculated. Baseline characteristics of the treatment and control groups are presented in Table 1. There were no significant differences between the 2 groups in any of the characteristics shown. Of the 174 children with known birth dates, 92.5% were younger than 9 y and the rest were between 9 and 11 y old. Few children were stunted or overweight, and almost none was underweight. Twenty-two percent of children in the treatment group and 14% in the control group had a DR:R ≥ 0.060, which indicated inadequate vitamin A liver stores. Anemia was present in 27% and 37% of the treatment and control groups, respectively, but only ≈ 18% in both groups had both anemia and low serum ferritin concentrations. Slightly more than 50% of the children in both groups had low serum zinc concentrations. None of the children had clinical symptoms of infection on the days blood was drawn, but subclinical infection was present in a few children: between 7% and 9% of children in both groups had an elevated CRP concentration only or an elevated AGP concentration only, whereas 2–3% had both elevated CRP and AGP concentrations.

**Sweet potato consumption and β-carotene intake**

Mean compliance with sweet potato consumption was 90 ± 8% and 89 ± 9% in the treatment and control groups, respectively. Only 11 children (12%) and 9 children (10%) in the treatment and control groups, respectively, were < 80% compliant; absence from school was the main reason for noncompliance. Five children in the treatment group and 2 in the control group said at some time during the intervention that they felt sick or did not want to eat the sweet potato. The β-carotene contents of 5 composite samples of boiled and mashed OFSP taken at evenly spread intervals during the intervention are shown in Table 2. The Resisto variety contains β-carotene almost exclusively, whereas the Bosbok WFPS variety is devoid of provitamin A carotenoids. A few other unidentified carotenoids in the Resisto variety were noted, but these compounds existed in negligible amounts. Thus, it was not necessary to identify and quantify these other carotenoids. The servings sizes of sweet potato in both treatment groups are shown in Table 3. The average amount of OFSP and WFSP served per day was similar between the 2 groups. Children in the treatment
group received an average of 12.375 μg β-carotene/d (1.1 RAE/d). OFSP provided 2.5 times the recommended dietary allowance of vitamin A for 4–8-y-old children, which is 400 μg RAE/d (19).

In the treatment group, 92% of children stated that they would like to eat OFSP every day of the week, 92% also found the taste acceptable, and 67% said that they would like to eat more than the serving size provided.

### Changes in vitamin A status

Mean baseline and postintervention values; mean changes in DR:R, DR dose response, and serum retinol concentrations; and estimated intervention effects are presented in Table 4. Mean values at baseline were not significantly different between the 2 groups. A significant intervention effect was found for the main values at baseline were not significantly different between the 2 groups. A significant intervention effect was found for the main

<table>
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<th>Control group (WFSP) (n = 89)</th>
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</tr>
<tr>
<td>Micronutrient indicator</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low serum retinol, &lt; 0.70 μmol/L (%)</td>
<td>71</td>
<td>73</td>
<td>0.739</td>
</tr>
<tr>
<td>Vitamin A status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal liver stores, DR:R &lt; 0.060 (%)</td>
<td>78</td>
<td>86</td>
<td>0.177</td>
</tr>
<tr>
<td>Insufficient liver stores, DR:R ≥ 0.060 (%)</td>
<td>22</td>
<td>14</td>
<td>0.118</td>
</tr>
<tr>
<td>Anemia, hemoglobin &lt; 115 g/L (%)</td>
<td>27</td>
<td>37</td>
<td>0.148</td>
</tr>
<tr>
<td>Low serum ferritin, &lt; 15 μg/L (%)</td>
<td>60</td>
<td>52</td>
<td>0.291</td>
</tr>
<tr>
<td>Anemia and low serum ferritin (%)</td>
<td>17</td>
<td>19</td>
<td>0.696</td>
</tr>
<tr>
<td>Low serum zinc, &lt; 10.7 μmol/L (%)</td>
<td>51</td>
<td>53</td>
<td>0.764</td>
</tr>
<tr>
<td>Elevated acute phase protein *</td>
<td>7</td>
<td>9</td>
<td>0.578</td>
</tr>
<tr>
<td>AGP &gt; 1.2 g/L (%)</td>
<td>8</td>
<td>9</td>
<td>0.787</td>
</tr>
<tr>
<td>Elevated CRP and AGP (%)</td>
<td>2</td>
<td>3</td>
<td>0.650</td>
</tr>
</tbody>
</table>

1 OFSP, orange-fleshed sweet potato (Resisto variety); WFSP, white-fleshed sweet potato (Bosbok variety); DR:R, ratio of 3,4-didehydroretinol to retinol; CRP, C-reactive protein; AGP, α1-acid glycoprotein.
2 Chi-square test unless indicated otherwise.
3 The birth date was not available for 1 child in the treatment group and for 3 children in the control group; thus, their age and anthropometric index could not be calculated.
4 x ± SD (all such values).
5 t test.
6 Proportion of children with height-for-age and weight-for-age z scores < −2 SDs of the median of the reference population (15).
7 Proportion of children with weight-for-height z scores > 2 SDs of the median of the reference population (15).
8 Proportion of children that satisfied the defined criteria; one child in each group was absent when blood was drawn at the end of the study.

### TABLE 2

<table>
<thead>
<tr>
<th>β-Carotene content of boiled and mashed orange-fleshed sweet potato (Resisto variety) in the composite samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composite sample</td>
</tr>
<tr>
<td>--------------------------------------------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>D</td>
</tr>
<tr>
<td>E</td>
</tr>
<tr>
<td>Average</td>
</tr>
</tbody>
</table>

1 All values are x ± SD of triplicate samples.
2 All values are x ± SD. RAE, retinol activity equivalents [12 μg β-carotene = 1 μg retinol = 1 RAE (19)].

### TABLE 3

<table>
<thead>
<tr>
<th>β-Carotene content provided during the intervention in the treatment and control groups¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composite sample</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

1 OFSP, orange-fleshed sweet potato (Resisto variety); WFSP, white-fleshed sweet potato (Bosbok variety); RAE, retinol activity equivalents [12 μg β-carotene = 1 μg retinol = 1 RAE (19)].
³ Average weight; x ± SD of 360 servings.
⁴ β-Carotene per serving: 124 g/100 g × 9980 μg β-carotene.
⁵ RAE per serving: 12 375 μg β-carotene/12.
liver stores in the treatment group relative to the control group ($P = 0.0203$). The proportion of children with normal vitamin A status, or adequate vitamin A liver stores ($\text{DR:R} < 0.060$), in the treatment group tended to increase (from 78% to 87%; $P = 0.096$) after intervention, whereas that in the control group did not change significantly (from 86% to 82%; $P = 0.267$). The proportions of children with normal liver stores were not used to test the intervention effect and within-group changes because the study was powered to test the intervention effect on $\text{DR:R}$. The change in $\text{DR:R}$ within each group was not significant, probably because the mean baseline $\text{DR:R}$ of the children indicated adequate vitamin A stores. There were no significant intervention effects for serum retinol and DR dose response. Serum retinol concentrations increased significantly from baseline in both groups. The proportion of children with a low serum retinol concentration ($<0.70 \mu\text{mol/L}$) after intervention decreased from 71% to 50% ($P = 0.001$) in the treatment group and decreased from 73% to 49% ($P = 0.001$) in the control group. The serum DR dose response increased significantly only in the control group.

**DISCUSSION**

This randomized controlled study showed that feeding $\beta$-carotene–rich OFSP of the Resisto variety in a primary school feeding program in a rural community improved vitamin A liver stores as measured with the MRDR test. The MRDR test (12) takes advantage of the vitamin A deficiency–dependent accumulation of apo-retinol binding protein (apo-RBP) in the liver (20) and is a better discriminator of intervention effects on vitamin A status than is serum retinol concentrations alone. The $\text{DR:R}$, which is not affected by subclinical infection (21, 22), showed an intervention effect, which indicated that $\beta$-carotene–rich OFSP improved vitamin A liver stores. The increase in the serum DR dose response in the control group indicated that apo-RBP accumulated in the liver as a result of inadequate endogenous liver retinol concentrations.

The MRDR test is more responsive to vitamin A intervention than are serum retinol concentrations when the supply of vitamin A is large enough to change overall vitamin A status (23). Serum retinol concentrations reflect recent vitamin A intakes (24). For the MRDR test to work correctly, ie, for it to pick up differences in vitamin A liver reserves, a washout period of $\geq 10$ d is allowed before the postintervention MRDR test is conducted. The washout period allows for the re-accumulation of apo-RBP in the hepatocytes of children who may not have changed to a normal vitamin A status during intervention with OFSP. This washout period may explain the lack of an intervention effect on serum retinol concentrations observed in this study because the dietary component of the serum retinol concentration would have been removed for 10 d before resampling. In another study done in South Africa, a biscuit fortified with $\beta$-carotene, providing 50% of the recommended dietary allowance for vitamin A for 1 y, resulted in an improvement in serum retinol concentrations in primary school children, but reverted to preintervention concentrations after a 10-wk period during which the fortified biscuits were not available (25).

The reason why serum retinol concentrations increased from baseline in both groups is unknown. Deworming to exclude helminthic infection as a confounder may have played a role. A study in Indonesian children showed that serum retinol concentrations markedly improved when children consuming meals containing plant sources high in $\beta$-carotene were dewormed (8). Others, however, have found that deworming and supplementing

### TABLE 4

Serum concentrations, changes, and intervention effects of vitamin A–status indicators in the treatment and control groups

<table>
<thead>
<tr>
<th></th>
<th>Treatment group (OFSP) ($n = 89$)</th>
<th>Control group (WFSP) ($n = 89$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DR:R (mol/mol)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>$0.040 \pm 0.028^{b}$</td>
<td>$0.038 \pm 0.024^{b}$</td>
</tr>
<tr>
<td>Postintervention</td>
<td>$0.036 \pm 0.019$</td>
<td>$0.042 \pm 0.025^{b}$</td>
</tr>
<tr>
<td>Change</td>
<td>$-0.004 (-0.009, 0.001)^{f}$</td>
<td>$0.004 (-0.001, 0.009)$</td>
</tr>
<tr>
<td><strong>DR dose response (nmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>$22.7 \pm 13.1$</td>
<td>$21.9 \pm 13.7^{f}$</td>
</tr>
<tr>
<td>Postintervention</td>
<td>$25.2 \pm 11.1$</td>
<td>$27.7 \pm 15.7^{f}$</td>
</tr>
<tr>
<td>Change</td>
<td>$2.6 (-0.1, 5.2)$</td>
<td>$5.9 (2.5, 9.3)^{f}$</td>
</tr>
<tr>
<td><strong>Retinol (µmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>$0.618 \pm 0.184$</td>
<td>$0.603 \pm 0.182$</td>
</tr>
<tr>
<td>Postintervention</td>
<td>$0.739 \pm 0.199$</td>
<td>$0.690 \pm 0.177$</td>
</tr>
<tr>
<td>Change</td>
<td>$0.121 (0.089, 0.153)^{g}$</td>
<td>$0.087 (0.052, 0.122)^{g}$</td>
</tr>
</tbody>
</table>

1. One child in each group was absent when blood was drawn at the end of the study. OFSP, orange-fleshed sweet potato (Resisto variety); WFSP, white-fleshed sweet potato (Bosbok variety); $\text{DR:R}$, ratio of $3,4$-didehydroretinol to retinol.
2. Determined 5 h after a dose of 2 mg ($7 \mu$mol) $3,4$-didehydroretinyl acetate.
3. $\bar{X} \pm SD$ (all such values).
4. $n = 88$.
5. $\bar{X}$; 95% CI in parentheses (all such values).
6. $^{d,e}$ Difference in mean change from baseline to postintervention between the treatment and control groups ($t$ test); $^{e, f}$ $P = 0.0203; ^{g, h}$ $P = 0.0130; ^{i, j}$ $P = 0.1535$.
7. $^{7,8}$ Significantly different from baseline within group ($t$ test for paired data); $^{7} P < 0.001; ^{8} P < 0.0001$. 

β-CAROTENE–RICH ORANGE-FLESHED SWEET POTATO 1085
children with 210 μmol vitamin A did not affect serum retinol concentrations (21). The increase in serum retinol concentration is dependent on the degree of intestinal helminth infection (8) and likely on the type of parasite. The present study did not assess the prevalence or degree of helminthic infection.

A seasonal effect on serum retinol concentrations in response to dietary changes, eg, when provitamin A–rich mangoes are available as reported in rural Gambian women (26), cannot be excluded, although it is unlikely. The baseline survey was conducted at the end of the summer season, whereas the postintervention survey was conducted during the winter season when provitamin A–containing foods are less available. The habitual diet of the children with regard to vitamin A and provitamin A intake during the intervention period was not controlled for. It was assumed that the habitual vitamin A intake was low, as was previously reported for rural areas in the same province (3, 11).

The bioavailability and bioconversion of provitamin A carotenoids depend on both the food matrix and host-related factors (27, 28). The β-carotene in orange-fleshed vegetables, such as the OFSP, does not play a role in photosynthesis and it is located in the cell chromoplasts (29), where it is found in lipid droplets or bound to a protein. The β-carotene in orange-fleshed vegetables is more readily released than that in dark-green leafy vegetables during cooking, thereby enhancing bioavailability. Between 3 g (8) and 5 g (30) fat per meal is required to ensure maximum carotenoid absorption. The sunflower seed oil added to the OFSP together with the fat content of the school meal provided ≥3 g fat.

The US Institute of Medicine recommendation that 12 μg β-carotene is equivalent to 1 μg retinol was used in this study (19). Bioefficacy, however, varies depending on vitamin A status and may be higher in vitamin A–deficient populations because such people are more efficient at converting provitamin A (31–34).

The low percentage of children with inadequate vitamin A liver stores (DR:R ≥ 0.060) at baseline was a limitation of the study. A bigger response may have occurred if a larger proportion of the study population had abnormal DR:R values from the onset. This finding shows that it is important to know the vitamin A status of the population before conducting intervention trials and to not rely solely on low serum retinol concentrations. If the study outcome had only relied on serum retinol concentrations, it would not have been possible to conclude anything regarding the efficacy of sweet potato in improving vitamin A status.

β-Carotene–rich foods are important for preventing vitamin A deficiency (31). A combination of orange fruit and squash is more effective in increasing serum retinol concentrations in anemic schoolchildren with marginal vitamin A status than was a combination of dark-green leafy vegetables and carrots (35). Consumption of meals containing β-carotene–rich red sweet potato also increased serum retinol concentrations in marginally vitamin A–deficient children (8). These findings may have been related to the type of β-carotene, because cis-isomers are less bioavailable and have less provitamin A activity than do the trans form (27, 28). The provitamin A in raw and boiled OFSP of the Resisto variety is almost exclusively β-carotene in the trans form (36).

OFSPs, which are naturally rich in β-carotene, are an excellent food source of provitamin A. These varieties can make a significant contribution to a viable long-term effective and sustainable food-based approach to prevent vitamin A deficiency in developing countries (6, 7). OFSP of the Resisto variety was successfully introduced into a home garden project that promoted the production and consumption of a variety of β-carotene–rich vegetables. It was shown that serum retinol concentrations in 2–5-y-old children improved within 20 mo of implementation (9).

Food diversification through the production of yellow-orange β-carotene–rich vegetables is seen as a viable long-term strategy to complement supplementation and fortification programs. High β-carotene–containing OFSP that provided ≈ 830 RAE/100 g cooked root was shown in this study to improve vitamin A status and to have the potential to control vitamin A deficiency in developing countries.

We gratefully acknowledge and thank the International Potato Center, Lima, Peru, for the opportunity to participate in the VITAA initiative; Frances Davidson, USAID, for her interest and support of this work; The Valley Trust, a nongovernmental organization, for assisting with the identification of a suitable school to participate in the study; the principal of the Ndunakazi Primary School for facilitating the meeting with educators and mothers; the MRC nutrition monitors for their invaluable support; the 7 study school’s governing body, principal, and Grade 1–3 educators for their cooperation and collaboration; the parents and the children who participated in the study; Martelle Marais, De Wet Marais, and Eldrich Harmse (Nutritional Intervention Research Unit) and the phlebotomist for their excellent technical support during blood sample collection and laboratory analyses; Sunette Laurie of the South African Agricultural Research Council, Vegetable and Ornamental Plant Institute (ARC-Rooedeplaat), for cultivating and supplying the sweet potatoes for this study; and Delia Rodriguez-Amaya, Departamento de Cienciia de Alimentos, Universidade Estadual de Campinas, SP, Brasil, under whose guidance the method for measuring β-carotene content in boiled and mashed OFSP by HPLC was developed.

PJvJ was the principal investigator responsible for all aspects of the study, with support from MF. All authors contributed to designing the study, interpreting the data, and writing the manuscript. SAT visited the laboratory of the Nutritional Intervention Research Unit and assisted in standardizing the HPLC method for analyzing serum DR and retinol. CJL was responsible for the statistical analysis. None of the authors had any conflict of interest with the funders of this study.

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Ascorbic acid supplementation and regular consumption of fresh orange juice increase the ascorbic acid content of human milk: studies in European and African lactating women

Synøve Daneel-Otterbech, Lena Davidsson, and Richard Hurrell

ABSTRACT

Background: Little is known about the influence of an increased intake of ascorbic acid (AA) on human milk AA output.

Objective: We aimed to compare human milk AA content in European and African women and to evaluate the influence of increased AA intake on human milk AA output.

Design: Apparently healthy lactating women were recruited. AA was analyzed by titration with 2,6-dichlorophenol-indophenol.

Results: Mean human milk AA was ≈50% lower (P < 0.001) in the African women (31 mg/kg; n = 171) than in the European women (63 mg/kg; n = 142). AA supplementation (1000 mg/d for 10 d) increased mean human milk AA from 19 to 60 mg/kg (P < 0.001) and from 60 to 70 mg/kg (P = 0.03) in 18 African and 10 European women, respectively. In 11 African women, mean human milk AA increased from 17 to 36 mg/kg (P < 0.001) after intake of 100 mg AA/d for 10 d. In African women, intake of 1 serving of orange juice per week had no significant effect, whereas 3 or 5 servings/wk (≈100 mg AA/serving) for 6 wk increased mean human milk AA from 16 to 32 mg/kg (n = 13) and from 21 to 46 mg/kg (n = 13), respectively (P < 0.001).

Conclusions: Human milk AA can be doubled or tripled by increased intake of AA in women with low human milk AA content at baseline. The response to a relatively high dose of AA was modest in European women in contrast with the 3-fold increase in mean human milk AA content in African women. These data indicate that human milk AA content is regulated.

KEY WORDS  Vitamin C, breastfeeding, infants, fruit juice, ascorbic acid

INTRODUCTION

Lactating women in developing countries have been reported to have significantly lower human milk ascorbic acid output than do women in industrialized countries (1–7). Human milk ascorbic acid content varies with maternal intake of ascorbic acid. Generally, lower maternal dietary intake of ascorbic acid and more pronounced seasonal variation in consumption of ascorbic acid–rich foods such as fruit and vegetables have been identified as major reasons for the low human milk ascorbic acid content of women living in resource-poor areas (4, 6, 8–10).

As a result of the large differences in the content of ascorbic acid in human milk between lactating women in different settings, the intake of ascorbic acid in exclusively breastfed infants varies widely. Although no cases of scurvy have been reported in breastfed infants, and the daily requirement for dietary ascorbic acid to prevent overt ascorbic acid deficiency in breastfed infants appears to be very low [7 mg/d; reviewed by the Institute of Medicine in 2000 (11)], the potentially negative influence of low ascorbic acid intake in early life related to the antioxidative properties of ascorbic acid has not been evaluated. Furthermore, human milk can be a major source of ascorbic acid in the overall diet of older infants consuming complementary foods, because traditional feeding practices in some settings limit the intake of ascorbic acid–rich foods during early life. For example, our recent study in Côte d’Ivoire clearly showed the importance of human milk as the major source of ascorbic acid in the diet of children aged 6–18 mo (12). Under these conditions, the child’s dietary intake of ascorbic acid is directly dependent on his or her mother’s human milk ascorbic acid output and therefore dependent on maternal intake of ascorbic acid. Ascorbic acid intake in the African infants and young children in our previous study varied widely and resulted in large variation in the molar ratios of ascorbic acid to iron in the overall diet (12).

Our findings in Côte d’Ivoire thus highlighted the need to evaluate sustainable approaches to increasing human milk ascorbic acid output in lactating women in resource-poor areas. Only limited information is available on the influence of increased intake of ascorbic acid during lactation, and we therefore initiated a series of studies to evaluate the effect of ascorbic acid supplementation and increased consumption of an ascorbic acid–rich food, orange juice.

The aims of the present study were to compare human milk ascorbic acid content of European and African lactating women and to evaluate the influence of an ascorbic acid supplement (1000 mg/d) in both study populations. In African women, we also evaluated the influence of a lower dose of ascorbic acid as a supplement (100 mg ascorbic acid/d) as well as the effect of...
regular consumption of fresh orange juice, served 1–5 times/wk (≈100 mg ascorbic acid/serving) for 6 wk. African women with relatively low human milk ascorbic acid content (<40 mg/kg) were enrolled into the longitudinal intervention studies.

SUBJECTS AND METHODS

Subjects

Apparently healthy, lactating women with apparently healthy infants older than 1 mo were recruited in Zurich, Switzerland, and in Abidjan, Côte d’Ivoire. Individual data on age, parity, date of birth, birth weight, and duration of breastfeeding were collected from each woman on the basis of recall or, in Abidjan, partly by using information from documents used for antenatal, prenatal, and infant health care (“carnet de santé pour la mère et l’enfant”). European women were recruited at meetings attended by lactating mothers. In Abidjan, lactating mothers were recruited at an information center that focused on infant nutrition at the National Institute of Public Health (Institut de Santé Publique). In addition, women were recruited by personal contacts in 2 low-socioeconomic areas (Atécoubé and Koumassi). To evaluate the effect of increased intake of ascorbic acid in women with different baseline ascorbic acid milk content, African women were recruited on the basis of an initial screening study. African women with milk ascorbic acid content <40 mg/kg were enrolled into studies 2, 4, and 5. All women enrolled into studies 2–5 were asked to not change their dietary habits or general lifestyle and to not consume ascorbic acid supplements (unless provided by the investigators) throughout the study.

The study protocol was reviewed and approved by the ethical committee at the University Hospital of Zurich, Switzerland, and by Institut National de Santé Publique in Abidjan. In addition, permission to implement study 5 was obtained from local authorities in Koumassi and Atécoubé. Women were informed about the aims and procedures of the study orally, and oral consent was obtained from each woman before the start of the study.

Study design

Five separate studies were implemented. Study 1 was a screening study to evaluate ascorbic acid in human milk collected from European and African women. One sample of human milk was collected from each woman. Study 2 was designed to evaluate the influence of an additional intake of 1000 mg ascorbic acid/d for 10 consecutive days in European and African women. One sample of human milk was collected every day from each woman. In study 3, a group of European women consumed 1000 mg ascorbic acid/d for 5 consecutive days and were followed for 35 d after discontinuation of the supplement. Three samples of human milk were collected during the 5-d supplementation. Additional samples were collected twice per week during the next 35 d. The influence of a smaller dose of ascorbic acid (100 mg ascorbic acid/d) for 10 consecutive days was evaluated in African women in study 4. One sample of human milk was collected every day from each woman. In study 5, a dietary intervention was implemented to evaluate the influence of 1, 3, or 5 servings of fresh orange juice (≈100 mg ascorbic acid/serving) per week for 6 consecutive weeks in African women. Samples of human milk were collected weekly during the supplementation (1 sample/wk from the women receiving 1 serving of orange juice per week and 2 samples/wk from the women served 3 or 5 servings of orange juice per week).

Ascorbic acid supplements

Effervescent tablets (1000 mg ascorbic acid; Redoxon, Roche Pharma AG, Reinach, Switzerland) were dissolved in water immediately before consumption. For preparation of the lower dose of ascorbic acid, individual doses of ascorbic acid (100 mg food-grade ascorbic acid; Merck, Dietikon Switzerland) were prepared and dissolved in water immediately before consumption. European women prepared and consumed their supplements at home daily, whereas in Abidjan, all supplements were administered by one of the investigators. All supplements were consumed between 0700 and 1200.

Dietary intervention

Fresh orange juice was used to increase dietary intake of ascorbic acid during study 5. Oranges were purchased in Abidjan, and each serving of orange juice (180 g) was prepared immediately before consumption by one of the investigators. It was estimated that 180 g orange juice would provide ≈70–100 mg ascorbic acid (37–54 mg/100 g orange juice) (13). Ascorbic acid in orange juice was analyzed by titration with 2,6-dichlorophenol-indophenol (14).

Human milk sampling and ascorbic acid analysis

The study protocol used during sampling and analysis of human milk samples was developed during a preliminary study (12). Human milk sampling could not be standardized, in particular during the fieldwork in Abidjan. Thus, milk samples were collected at different times after the last breastfeeding, and the amount of milk expressed at each sampling varied. Furthermore, because of the lack of access to sophisticated analytic equipment such as HPLC in the laboratory in Abidjan and concern over potential losses of ascorbic acid during transport of frozen samples to Zurich, we used a simple analytic technique based on titration with 2,6-dichlorophenol-indophenol (14) to measure ascorbic acid in the present study. A limitation of this method is that it measures only reduced ascorbic acid and not total ascorbic acid. According to information from previous studies, the content of dehydroascorbic acid compared with reduced ascorbic is low in human and cow milk (15, 16). In addition, because a major aim of the present study was to compare data before and after interventions to increase ascorbic acid intake, the methodologic limitations of the technique based on titration with 2,6-dichlorophenol-indophenol are considered to be of limited importance.

Briefly, data from the preliminary study (12) showed no statistically significant influence of the meta-phosphoric acid (MPA) concentration in human milk samples [2%, 4%, or 6% MPA; n = 9; P = 0.245 (multivariate analysis with repeated measures)] or on the amount of milk used during the analysis (1, 2, 3, 5, or 10 g/sample; n = 9; P = 0.455, multivariate analysis with repeated measures) on ascorbic acid content. In addition, ascorbic acid content was not significantly different between foremilk and hindmilk (n = 25; P = 0.95, paired Student’s t test). Human milk samples (MPA concentration of 2%) were shown to be stable during storage at 4 °C for 24 h (n = 13; P = 0.926, multivariate analysis with repeated measures) and at −20 °C for 6 mo (P = 0.384, multivariate analysis with repeated measures).
We also compared ascorbic acid content in human milk analyzed by titration with 2,6-dichlorophenol-indophenol and by HPLC (12). The results were significantly different ($P < 0.001$, paired Student’s $t$ test; $n = 26$): $32.5 \pm 15.9$ compared with $26.3 \pm 15.1$ mg/kg, respectively. The correlation between data based on the 2 analytic methods was significant (Pearson correlation coefficient = 0.99, $P < 0.001$). Significantly larger differences were found at higher human milk ascorbic acid contents than at lower ascorbic acid contents (Pearson correlation coefficient = 0.47, $P < 0.02$). The CV based on human milk samples analyzed by titration with 2,6-dichlorophenol-indophenol in triplicate ($n = 100$) was $1.3 \pm 1.3\%$. Analysis of human milk collected on 3–7 consecutive days showed no significant difference (multivariate analysis, repeated measures) in ascorbic acid content (titration with 2,6-dichlorophenol-indophenol): 3 consecutive days ($n = 63; P = 0.334$), 5 consecutive days ($n = 22; P = 0.260$), and 7 consecutive days ($n = 7; P = 0.878$).

In the present study, all samples were collected between 0700 and 1200, before intake of ascorbic acid supplements or orange juice. In studies 1–4, women used battery operated or electric breast pumps (Medela AG, Baar, Switzerland). In study 5, women expressed milk manually. All milk samples were collected directly into preweighed, opaque polypropylene containers (35 mL) containing a preweighed amount of 10% MPA solution (Merck) to prevent autooxidation of ascorbic acid during storage and analysis (14). Fresh solutions of MPA were prepared weekly, stored refrigerated, and protected against light. MPA and human milk were mixed by gently shaking the container. The amount of human milk collected was $14 \pm 5$ g/sample (studies 1–5), and the MPA concentration was in the range of 2.4–5.2% before freezing. Immediately before analysis, the MPA concentration was adjusted to 2% in all samples. Samples were kept cool during transport to the local laboratory and were stored at $-20\,^\circ C$ until analyzed.

In studies 2–5, two samples of human milk were collected on separate days before the intervention started, and the mean ascorbic acid content of these two samples is referred to as the baseline value. At other samplings, one spot sample was collected (see the information about sampling frequency in each study in the “Study design” section).

All samples were analyzed by one of the investigators. Samples collected in Abidjan were analyzed at Centre Suisse de Recherches Scientifiques, and samples collected in Zurich were analyzed at the Laboratory for Human Nutrition in Rüschlikon. Milk samples were thawed at room temperature immediately before analysis. All samples were analyzed within 2 wk, in duplicate. Results are presented as mg ascorbic acid/kg human milk.

**Statistical analysis**

Maternal age and duration of lactation were compared in European and African women by use of unpaired Student’s $t$ tests. Parity was compared in the 2 study populations by use of the Mann-Whitney $U$ test (nonparametric test). The distribution of human milk ascorbic acid content was evaluated by the Kolmogorov-Smirnov test and the QQ-Normal-Distribution-Plot.

Different statistical methods were used to evaluate data in the separate studies. In study 1, the data were evaluated by unpaired Student’s $t$ tests. In study 2, changes in human milk ascorbic acid content (day to day) during the intervention were evaluated by using a general linear model (repeated measures) including repeated contrasts. Paired Student’s $t$ test was used to compare human milk ascorbic acid content at baseline with human milk ascorbic acid content at the end of the study (day 11). In study 3, human milk ascorbic acid content during the follow-up period was evaluated for each woman through visual inspection of the scatter plot. A general linear model (repeated measures) with simple contrast and ascorbic acid content at baseline as the reference category was used to compare milk ascorbic acid content at 3 time points: at baseline, after the completion of supplementation (day 6), and at the time when milk ascorbic acid content had returned to baseline. In study 4, paired Student’s $t$ tests were used to compare human milk ascorbic acid content at baseline with human milk ascorbic acid content at the end of the study (day 11). Paired Student’s $t$ tests were used to compare human milk ascorbic acid content at baseline with human milk ascorbic acid content at the end of study 5 and to evaluate whether changes in human milk ascorbic acid content were different from zero. Baseline values were compared by one-way ANOVA with post hoc Tukey’s test. Analysis of covariance (with baseline as a covariate) was used to analyze the data from the 3 intervention groups in study 5.

$P$ values $< 0.05$ are referred to as indicating significance. A commercial statistical software package (SPSS 12.0 for WINDOWS; SPSS Inc, Chicago, IL) was used to evaluate the data.

**RESULTS**

The African women were significantly younger ($25 \pm 5.5$; range: 16–41 y), had higher parity (2.4 ± 3.3; range: 1–9), and had been breastfeeding for longer periods of time (8.2 ± 3.3 mo; range: 1.0–18.2 mo) than the European women [age, 33 ± 3.2 y (range: 22–43 y); parity, 1.5 ± 0.5 (range: 1–4); and breastfeeding duration, 4.5 ± 1.9 mo (range: 1.0–21.8 mo); $P < 0.01$; studies 1–5].

Human milk ascorbic acid values were normally distributed, and the results are presented as means ± SDs. No significant difference in human milk ascorbic acid content was observed between samples collected during the screening study and samples collected at baseline in 65 African women ($P = 0.451$, paired Student’s $t$ test; studies 2–5).

**Study 1**

Human milk ascorbic acid content was significantly lower in the African women (31 ± 15 mg/kg; $n = 171$) than in the European women (63 ± 14 mg/kg; $n = 142$; $P < 0.001$, unpaired Student’s $t$ test).

**Study 2**

Intake of 1000 mg ascorbic acid/d for 10 d resulted in significantly increased human milk ascorbic acid content in both the European and the African women. In 10 European women, human milk ascorbic acid increased from 60 ± 12 mg/kg at baseline to 70 ± 16 mg/kg after intake of a cumulative dose of 10 000 mg ascorbic acid ($P = 0.03$, paired Student’s $t$ test; Figure 1). Corresponding values for the 18 African women in this study were 19 ± 16 and 60 ± 11 mg/kg ($P < 0.001$, paired Student’s $t$ test; Figure 1). In the African women, ascorbic acid content increased significantly from day to day during the first few days of the intervention (general linear model, repeated measures). However, after intake of a cumulative dose of 4000 mg ascorbic...
acid, no further increase in ascorbic acid content was observed. In the European women, no significant day-to-day increase was observed.

Study 3

Human milk ascorbic acid content increased from 70 ± 11 to 82 ± 11 mg/kg after intake of 1000 mg ascorbic acid/d for 5 d (P < 0.001, paired Student’s t test) in 17 European women. After discontinuation of the supplementation, human milk ascorbic acid content was not significantly different from baseline for the first time (68 ± 12 mg/kg; P = 0.303, general linear model, repeated measures) after 21 d.

Study 4

Human milk ascorbic acid content increased from 17 ± 6.5 to 36 ± 8.0 mg/kg (P < 0.001, paired Student’s t test) in 11 African women after intake of 100 mg ascorbic acid/d for 10 d.

Study 5

Mean ascorbic acid content per serving of orange juice (180 g) was 105 mg (range: 72 to 159 mg; n = 15). Baseline milk ascorbic acid content was significantly higher in the African women served one glass of orange juice per week (23 ± 5.3 mg/kg) than in the women consuming 3 servings of orange juice per week (16 ± 6.0 mg/kg; P = 0.02, one-way ANOVA with post hoc Tukey’s test). Baseline milk ascorbic acid was not significantly different in the African women consuming 5 servings of orange juice/wk (21 ± 4.3 mg/kg) than in the women consuming 1 or 3 servings/wk. At the end of the study, human milk ascorbic acid content was 26 ± 7.1 mg/kg (1 serving/wk), 32 ± 6.9 mg/kg (3 servings/wk), and 46 ± 6.2 mg/kg (5 servings/wk). Changes in human milk ascorbic acid content were not significantly different from zero in the women consuming one serving of orange juice per week (P = 0.75, paired Student’s t test), whereas human milk ascorbic acid content was significantly different from zero in the other 2 intervention groups at the end of the study (P < 0.001, paired Student’s t test).

Analysis of covariance (with baseline as a covariate) showed a significant influence of baseline values (P = 0.0041) and study group (P < 0.001), whereas their interaction was not significant. The main effect model with polynomial contrast showed the linear term to be significant for the study group (P < 0.001); the estimated effect was 11.8 mg/kg per 2 servings of orange juice/wk. The parameter estimate for baseline was −0.5 mg/kg. The change in human milk ascorbic acid content during the 6-wk dietary intervention period (adjusted for baseline ascorbic acid content) is presented in Figure 2.

DISCUSSION

Mean human milk ascorbic acid content was ≈50% lower in the African women (31 mg/kg; n = 171) than in the European women (63 mg/kg; n = 142) in the present study. These results provide additional information on the significant difference in human milk ascorbic acid output in women living in different settings. A striking difference between the 2 study populations was noted when evaluating the distribution of the data: 29% of the African women had very low human milk ascorbic acid output, 20–29 mg/kg, whereas none of the milk samples expressed by European women had such low ascorbic acid content. Although we did not collect data on dietary intake in all women participating in the study, preliminary data based on a subsample of subjects indicate a significant difference in ascorbic acid intake between African women living in Abidjan (mean intake: 22 mg/d) and European women living in Zurich (mean intake: 106 mg/d) (12). Furthermore, the importance of ascorbic acid intake on human milk ascorbic acid content was clearly shown in the subsequent intervention studies in both study populations.
Although earlier studies reported on the influence of increased intake of ascorbic acid in women living in developing countries, limited information is available on the effect of increased intake of ascorbic acid on human milk output in Western women. Because previous intervention studies in lactating women living in industrialized countries reported no effect of smaller doses of ascorbic acid (20–200 mg/d; 2, 3, 17) on human milk ascorbic acid content, we investigated the effect of a relatively high dose of ascorbic acid (1000 mg/d) in the present study. Intake of supplements providing 1000 mg per dose is not uncommon in industrialized countries, and the dietary supplement used in the present study is available over the counter in Switzerland.

To our knowledge, only one previous study included supplementation of lactating women with 1000 mg ascorbic acid/d; this study reported no significant influence on human milk ascorbic acid output (18). These results are not surprising because the intervention was short-term (2 d) and only 5 women participated in the study. Our data show that supplementation with 1000 mg ascorbic acid/d for 10 d increases mean human milk ascorbic acid significantly—from 60 to 70 mg/kg—in European women (P = 0.02). However, although the effect on human milk ascorbic acid output was significant, the overall increase was modest compared with the effect observed in the African women. Mean milk ascorbic acid increased 3-fold in the 18 African women participating in the supplementation study: from 19 mg/kg at baseline to 60 mg/kg on day 11 (P < 0.001). In addition to the pronounced overall effect of ascorbic acid supplementation on human milk ascorbic acid content, a significant day-to-day effect was observed during the early phase of the intervention in the African women; no such effect was found in the European women. Clearly, the response to ascorbic acid supplementation differed in the 2 study groups and, although the results from the present study do not provide any information on the mechanisms involved, the data indicate that human milk ascorbic acid content is regulated.

Earlier studies suggested that there is an upper limit to the ascorbic acid secretion by the mammary gland, presumably related to the saturation of the mammary tissue with the vitamin (18–20). The considerable interindividual variability of the upper limit of human milk ascorbic acid secretion by the mammary gland—and therefore the presumably large individual variation in the degree of saturation of mammary tissue—was emphasized in the previous study by Pratt et al (19) and also indicated in the present study (Figure 1). The mechanism or mechanisms regulating ascorbic acid saturation of the mammary tissue and secretion of the vitamin by the mammary gland are not known. We did not monitor urinary excretion of ascorbic acid in the present study, but it can be assumed that a large proportion of absorbed ascorbic acid was lost in urine (at intakes that raised plasma concentrations above the renal threshold). Fluctuations in human milk ascorbic acid content have been shown to be slower and less pronounced than the response in urinary excretion (18, 19). The potential influence of differences in the fractional absorption of ascorbic acid on human milk ascorbic acid output is not known, and we are not aware of any data evaluating differences in the fractional absorption of ascorbic acid related to habitual intake of ascorbic acid (and thus differences in body stores of ascorbic acid). Fractional absorption of ascorbic acid is dose dependent and has been reported to fall from 70–90% to ≈50% or less with doses >1000 mg/d (21).

After the initial supplementation study in European women, we also evaluated the effect of a shorter intervention (study 3) and monitored the decrease in human milk ascorbic acid output after discontinuation of the supplement. In this study group of European women, mean ascorbic acid milk output increased from 70 mg/kg at baseline to 82 mg/kg (P < 0.001) after intake of 1000 mg ascorbic acid/d for 5 d, and human milk ascorbic acid content had returned to values not significantly different from baseline values after 21 d. These results indicate that the body pool of ascorbic acid increased during the 5-d supplementation and that storage ascorbic acid was excreted in human milk after the supplementation was discontinued.

Clearly, high-dose ascorbic acid supplements are effective in increasing human milk ascorbic acid output in both European and African lactating women. However, the usefulness of this approach is obviously limited from a public health perspective. A more sustainable approach would be to encourage increased dietary intake of ascorbic acid by consumption of locally available ascorbic acid–rich foods. We therefore evaluated the influence of increased intake of fresh orange juice on human milk ascorbic acid output in African women. One serving of orange juice per week had no significant effect on human milk ascorbic acid output, but the beneficial effect of regular consumption of fresh orange juice 3 or 5 times/wk (≈100 mg ascorbic acid/serving) for 6 wk was clearly shown. Mean human milk ascorbic acid output increased ≈2-fold: from 16 to 32 mg/kg and from 21 to 46 mg/kg in the 2 study groups (P < 0.001 compared with zero difference, paired Student’s t test). The results from this dietary intervention also showed the dose effect of ascorbic acid on human milk ascorbic acid content (P < 0.001, analysis of covariance with baseline as a covariate; Figure 2). For comparison, we also evaluated the effect of a smaller dose of ascorbic acid as a supplement (100 mg ascorbic acid/d) for 10 d in 11 African women. At the
end of the study, mean human milk ascorbic acid content had increased =2-fold: from 17 to 36 mg/kg (P < 0.001). Thus, a doubling of human milk ascorbic acid content can be achieved by intake of relatively low doses of the vitamin, either as low-dose supplements or as ascorbic acid–rich fruit juice.

In conclusion, ascorbic acid supplementation with a relatively high dose of ascorbic acid (1000 mg/d for 10 d) increased human milk ascorbic acid output in both European and African women. Although significant, however, the overall effect was modest in well-nourished European women in contrast with the 3-fold increase in mean human milk ascorbic acid content observed in African women. These results indicate that human milk ascorbic acid content is regulated and are in agreement with previous observations (5, 18–20).

With lower doses of ascorbic acid, in the form of dietary supplements or as ascorbic acid–rich fruit juice, human milk output can be doubled in African women with relatively low human milk ascorbic acid content at baseline. The results from this study highlight the importance of encouraging regular consumption of ascorbic acid–rich foods by lactating mothers to ensure adequate human milk ascorbic acid output. Apart from the obvious importance of human milk as the sole source of ascorbic acid in exclusively breastfed infants, human milk can be an important source of ascorbic acid in the diet of breastfed older infants and young children consuming complementary foods. Although our recent study in Bangladesh did not show any significant difference in iron bioavailability from a traditional complementary food consumed with water or with human milk (22), further studies are needed to evaluate the effects of human milk as a source of ascorbic acid on iron bioavailability from less inhibitory meals.

We are indebted to all the women who participated in this study. The excellent technical assistance of Tina Adjara (Abidjan) and Karin Rustmeier (Zurich) during the study is gratefully acknowledged. We are grateful for the use of facilities at Institut National de Santé Publique and Centre Suisse de Recherches Scientifiques in Abidjan. Finally, we thank Christoph Buser for advice on the statistical analyses.

All authors contributed to the study design. SD-O was responsible for the implementation of the study and for data collection and analysis. LD was responsible for data analysis and for the preparation of the manuscript. SD-O and RH contributed to the preparation of the final manuscript. None of the authors had any conflicts of interest.

REFERENCES

Supplementation of infant formula with long-chain polyunsaturated fatty acids does not influence the growth of term infants

Maria Makrides, Robert A Gibson, Tuesday Udell, Karin Ried, and the International LCPUFA Investigators

ABSTRACT
Background: Adequate growth is an important indicator of health and well-being in infants.
Objective: Our objective was to determine the effect of supplementing infant formula with long-chain polyunsaturated fatty acids (LCPUFAs) on the growth of term infants.
Design: Using the methodology outlined by the Cochrane Collaboration, we reviewed all known randomized controlled trials that involved LCPUFA supplementation of infant formula fed to term infants. Outcome measures were weight, length, and head circumference. Original data obtained from the investigators of published trials were used. Outcomes were analyzed with fixed-effects or random-effects model meta-analyses and were reported as weighted mean differences with 95% CIs.
Results: We identified 14 eligible trials that had data available for meta-analysis (1846 infants). Trial quality was generally high. Meta-analysis showed no significant effect of LCPUFA supplementation on infant weight, length, or head circumference at any assessment age. Similarly, subgroup analyses showed that supplementation with only n-3 LCPUFAs (no arachidonic acid) had no significant effect on infant weight, length, or head circumference. The source of LCPUFA supplementation (phospholipid or triacylglycerol) also did not significantly affect infant growth.
Conclusion: We found no evidence that LCPUFA supplementation of infant formula influences the growth of term infants in either a positive or a negative way. Am J Clin Nutr 2005;81:1094–1101.

KEY WORDS Systematic review, meta-analysis, docosahexaenoic acid, DHA, arachidonic acid, AA, infant, growth

INTRODUCTION
Growth is the cornerstone assessment of nutritional health and well-being in preverbal children. Growth failure can be one of the first clinical signs that indicate underlying pathology, whereas growth excess may indicate predisposition to obesity and associated chronic health problems (1, 2). Understanding the nutritional factors that alter growth are of interest to health workers and public health authorities alike.
An important change to the composition of infant formula in the past 5 y has been the addition of long-chain polyunsaturated fatty acids (LCPUFAs). The key LCPUFAs added have been docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6). Both DHA and AA are found in human milk, and infants fed unsupplemented formulas have lower concentrations of DHA and AA in their plasma and erythrocytes than do infants who are breastfed or fed supplemented formulas (3, 4). Some randomized controlled trials have shown that infants fed formulas with DHA alone or DHA in combination with AA have better performance in visual and developmental tests than do unsupplemented infants (5-9). However, it is also recognized that DHA and AA are integral structural components of all cells and may, therefore, have other health effects.
The effect of DHA and AA on infant growth has been somewhat controversial. Some of the early trials (which used fish oil containing only n-3 LCPUFA) suggested that preterm infants fed formulas supplemented with n-3 LCPUFA alone weighed less and were shorter than preterm infants fed standard, unsupplemented formulas (10-12). Later trials that intervened with formulas containing both DHA and AA have shown positive effects of supplementation on weight and length gains (13-16) as well as negative effects on linear growth (17). Although most trials that involved term infants have shown no effect of LCPUFA supplementation on growth, the observations in preterm infants have resulted in significant debate and raised many questions about the type and source of LCPUFA supplementation for both term and preterm infants. It has also resulted in many formulas for term infants being supplemented with a balance of DHA and AA that favors n-6 LCPUFA.

This paper reports the systematic review and meta-analysis of growth in randomized controlled trials that involved LCPUFA interventions in term infants. Subgroup analyses assessed whether there were differential effects of supplementation on growth depending on the type and source of LCPUFAs and whether there were differential effects of LCPUFA supplementation between boys and girls because of their differing growth patterns. A separate paper will report the meta-analysis that relates to growth outcomes of preterm infants.

1 From the Child Health Research Institute, Women’s and Children’s Hospital, North Adelaide, Australia (MM, RAG, and KR); the Department of Paediatrics, University of Adelaide, Adelaide, Australia (MM and RAG); and the Department Paediatrics and Child Health, Flinders University and Medical Centre, Adelaide, Australia (RAG and TU).
2 Supported by grants from Wyeth Nutrition; The Financial Markets for Children, Australia; New Zealand Milk; and Senior Research Fellowships from the National Health and Medical Research Council of Australia (to MM and RAG).
3 Reprints not available. Address correspondence to M Makrides, Applied Nutrition Group, Child Health Research Institute, Women’s and Children’s Hospital, 72 King William Road, North Adelaide, SA 5006, Australia. E-mail: makridesm@mail.wch.sa.gov.au.
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SUBJECTS AND METHODS

Search strategy

The Medline database and the proceedings of relevant conferences known to us were searched for randomized trials of dietary LCPUFA intervention in term infants. The following terms were used to search the Medline database from 1960 to July 2004 limited to randomized clinical trials: fatty acid, unsaturated; omega fatty acid; or n-3 fatty acid. The reference lists of retrieved publications were also searched for relevant trials. The last literature search was completed in July 2004. A total of 21 trials were evaluated for inclusion (5, 7, 8, 18–35).

Selection of trials

Trials that involved term infants were eligible for inclusion if they had a randomized design, growth was reported as an outcome measure, the LCPUFA intervention commenced within 14 d of birth, and the test diets were fed for at least 12 wk. Fifteen of the original 21 trials met these criteria (Figure 1). Two studies were excluded because they only assessed the effect of LCPUFA supplementation on infant blood concentrations of LCPUFAs (35, 36), 1 because the study started when the infants were >14 d old (33), 2 because the intervention diets were fed for <12 wk (32, 34), and 1 did not involve an LCPUFA intervention (37). One trial was subsequently excluded because mean weight, length, and head circumference data were not available from either the paper or the investigators (31). Therefore, a total of 14 trials were included in the meta-analysis (Figure 1).

Data abstraction and collection

The characteristics of included studies and the data were abstracted independently by 2 investigators. As mean weight, length, and head circumference data were not often reported in publications, investigators were contacted to provide data that were incomplete. Growth data from the included trials were abstracted on an intention-to-treat basis. Investigators were also asked to provide data separately for boys and girls, and we retrieved this information for 12 of 14 trials.

Financial support to conduct the systematic review was obtained from industry, charitable organizations, and government sources. The funding agencies had no influence on the abstraction and analysis of the data. Data from multicenter, industry-sponsored trials were supplied by Nutricia, Mead Johnson, and Abbott Laboratories.

Analysis

We compared the weight, length, and head circumference of LCPUFA-treated with untreated infants at 4 and 12 mo of age. These primary time points were chosen because 4 mo often represents the end of feeding formula as a sole source of nutrition and 12 mo often coincides with the end of formula feeding. When data were available, weight, length, and head circumference of infants were also compared at 3, 6, and 9 mo of age. Meta-analyses were computed by using the Metview Program available in REVIEW-MANAGER 4.1 (Cochrane Collaboration). A fixed-effects model was used when there was no significant heterogeneity between studies, and a random-effects model was used when significant heterogeneity existed. Each study was weighted in the meta-analysis according to the SD and number of infants. Subgroup analyses were conducted to determine whether there were differential effects according to sex, type, and source of LCPUFA supplementation. For example, we compared weight, length, and head circumference of infants treated with only n-3 LCPUFA with the control group, infants fed LCPUFAs from phospholipid sources with the control group, and infants fed LCPUFAs from triacylglycerol sources with the control group.

RESULTS

Summary of included studies

A total of 14 trials conducted across Europe (n = 6), North America (n = 6), and Australia (n = 2) were included in our review, and these trials are summarized in Table 1 (5, 7, 8, 18–30). All trials reported a randomized design, but it was not always clear from the published papers whether there was adequate concealment of allocation. All trials were placebo-controlled and compared standard cow milk–based formula with no LCPUFAs with equivalent formulas containing LCPUFAs. The energy and protein concentrations of test formulas across all trials were comparable and approximated 280 kJ/100 mL and 1.5 g/100 mL, respectively. Doses of n-3 LCPUFA in the supplemented formulas ranged from 0.1% to 1.0% of total fatty acids, and the dose of n-6 LCPUFA ranged from 0 to 0.72% of total fatty acids (Table 1). LCPUFA supplements were from a variety of sources, including fish oils, egg phospholipid fractions, egg triacylglycerol fractions, and algal and fungal oils. Eleven trials clearly reported that both parents and trial assessment staff members were blinded to each infant’s group allocation (5, 7, 8, 18–22, 25–29). Most trials intervened with their test diets

FIGURE 1. Randomized controlled trials (RCTs) identified and used in the systematic review and meta-analysis. Ref, reference; LCPUFA, long-chain polyunsaturated fatty acid.
through to 4 mo of age (5, 7, 8, 18–27, 29, 30) and also assessed the growth of infants at this age (5, 7, 8, 18–24, 27, 30). Six of the included trials reported <20% attrition of randomly assigned infants through to 4 mo of age (5, 7, 18, 19, 22, 27, 30) (Table 1).

Primary analysis

Most trials and the majority (1050 of 1662, 63%) of infants were assessed at 4 mo of age. At this time point the summary statistic indicated no differences in weight, length, or head circumference between infants allocated to either control or LCPUFA-supplemented formulas (see diamond symbols, Figures 2–4), despite individual studies that reported statistically significant effects on head circumference. Note that at 4 mo all included trials were actively feeding the test formulas, and infants were likely to have received few other sources of dietary LCPUFAs (38). Similarly, at 12 mo of age no differences were observed in the summary statistic for weight, length, or head circumference.
circumference between groups, although 1 study reported an effect on weight at this time (Figures 2–4). However, by this stage only 4 trials (653 infants) continued intervention with the test formulas. Two included trials had no growth measurements either at 4 or 12 mo but had measured growth at either 3 mo (29) or 6 and 9 mo (25, 26). Comparisons of weight, length, and head circumference at these ages also revealed no differences between LCPUFA-treated infants and control infants. Overall, mean weight, length, and head circumference from the meta-analysis at 3, 4, 6, 9, and 12 mo of age approximated the 50th percentiles on standardized growth charts (39, 40).

Subgroup analyses

Six trials intervened with a formula that contained only n–3 LCPUFA, and all of these trials also assessed growth at 4 mo of age (Table 1). Weight, length, and head circumference at 4 and 12 mo of age did not differ between groups (Figures 2–4). No consistent pattern was observed to indicate that dose of n–3 LCPUFA influenced weight, length, or head circumference. No differences were observed in weight, length, or head circumference between the groups at either 4 or 12 mo of age in the subgroup analyses related to sex, phospholipid sources of LCPUFAs, or triacylglycerol sources of LCPUFAs (data not shown).

![FIGURE 2. Meta-analysis forest plots for infant weight for all trials and for the subgroups intervening with only n–3 long-chain polyunsaturated fatty acid (LCPUFA). Trials are displayed according to increasing dose of n–3 LCPUFA, regardless of the presence of n–6 LCPUFA. Trials marked with an asterisk (*) had ceased dietary interventions at the indicated assessment. WMD, weighted mean difference.](image-url)
DISCUSSION

Our paper is the most comprehensive meta-analysis of the effect of LCPUFA supplementation of infant formula on the growth of term infants to date. It includes unreported details from published studies (most trials did not publish mean growth data for boys and girls) as well as data from the largest trial that are only available in abstract form. Most trials were conducted to a high standard, all enrolled infants in the first 2 wk of life, and all but one fed the test formulas until infants were at least 4 mo. This high degree of compatibility between trial protocols gave us confidence in the legitimacy of combining the data. Because studies were conducted in different countries with different population groups, the results of the meta-analysis can be generally applied to formula-fed infants. The main difference between the trial designs was in the type and source of LCPUFA supplementation tested, and this allowed us to statistically explore the effectiveness of a range of fatty acid types and sources through subgroup analyses.

The combined data show that no effect of LCPUFA supplementation of infant formula was observed on the growth of term infants at any age. This observation was not influenced by the type of supplementation (n-3 LCPUFA alone or n-3 + n-6

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<th>Time point</th>
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<th>LCPUFA n</th>
<th>Control n</th>
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<th>Weight (%)</th>
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</tr>
<tr>
<td>Aasen 1997</td>
</tr>
<tr>
<td>Carlson 1999</td>
</tr>
<tr>
<td>Agostoni</td>
</tr>
<tr>
<td>Birch</td>
</tr>
<tr>
<td>Makrides 1998</td>
</tr>
<tr>
<td>Makrides 1995</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

| 12 mo                         |
| Aasen 1997                    | 43        | 43        | 75.38 (3.02)      | 20.3       | -0.02 [-0.57, 0.53] |    |
| Carter                          | 20        | 20        | 75.45 (2.25)      | 23.7       | 0.85 [-1.21, 0.29]  |    |
| Makrides 1999                 | 44        | 44        | 75.23 (3.51)      | 19.5       | -1.23 [-2.79, 0.32]|    |
| Makrides 1995                 | 12        | 11        | 75.22 (2.73)      | 16.5       | 0.92 [0.92, 1.84]   |    |
| Total                         | 99        | 99        |                   | 100.0      | -0.40 [-1.15, 0.35]| 0.3 |

FIGURE 3. Meta-analysis forest plots for infant length for all trials and for the subgroups intervening with only n-3 long-chain polyunsaturated fatty acid (LCPUFA). Trials are displayed according to increasing dose of n-3 LCPUFA, regardless of the presence of n-6 LCPUFA. Trials marked with an asterisk (*) had ceased dietary interventions at the indicated assessment. WMD, weighted mean difference.
LCPUFA), the source of supplementation (triacylglycerol or phospholipid), or sex. Our results are also consistent with the Cochrane systematic review that assessed the effect of LCPUFA interventions on the outcomes of term infants, although the Cochrane review contained less growth data and undertook a less extensive analysis (41). Because the relative merits of the outcomes of systematic reviews compared with individual adequately powered trials are often discussed, our observations are particularly noteworthy because there is congruency between the results of individual well-powered trials [7 of 14 included trials according to independently published criteria (42)] and the results of the systematic review.

One of the most hotly debated issues that relates to LCPUFA supplementation of infant formula is whether n-3 LCPUFA could be added without a source of AA. Much of this debate stems from the early observations of growth deficits in preterm infants who received formulas that contained only n-3 LCPUFA compared with control formulas (10–12). It was hypothesized that the depression of plasma AA caused by dietary n-3 LCPUFA supplementation may be a factor that contributes to the growth deficit because both observational data (43) and 1 randomized trial (44) indicated an association between plasma AA and weight and length. However, in term infants there is no evidence of any reduction in weight, length, or head circumference.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Study</th>
<th>LCPUFA n</th>
<th>mean (SD)</th>
<th>Control n</th>
<th>mean (SD)</th>
<th>WMD (95% CI Fixed)</th>
<th>Weight (%)</th>
<th>WMD (95% CI Fixed)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All trials</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 mo</td>
<td>Carlson 1996</td>
<td>20</td>
<td>41.35 (0.78)</td>
<td>22</td>
<td>41.48 (0.82)</td>
<td>9.3</td>
<td>-0.13 [-0.31, 0.05]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Auestad 2001</td>
<td>121</td>
<td>41.83 (1.18)</td>
<td>104</td>
<td>41.76 (1.22)</td>
<td>17.7</td>
<td>0.01 [-0.34, 0.36]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Innis</td>
<td>68</td>
<td>41.52 (1.20)</td>
<td>69</td>
<td>41.45 (1.43)</td>
<td>6.3</td>
<td>0.12 [0.43, 0.67]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Auestad 1997</td>
<td>89</td>
<td>41.63 (1.12)</td>
<td>45</td>
<td>41.96 (1.06)</td>
<td>14.6</td>
<td>-0.23 [-0.62, 0.16]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Carlson 1999</td>
<td>212</td>
<td>41.59 (0.24)</td>
<td>104</td>
<td>41.76 (1.22)</td>
<td>26.3</td>
<td>0.20 [0.09, 0.31]</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Decorti</td>
<td>9</td>
<td>41.70 (0.95)</td>
<td>7</td>
<td>42.21 (1.14)</td>
<td>2.0</td>
<td>1.49 [0.44, 2.54]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Agostoni</td>
<td>31</td>
<td>41.21 (1.09)</td>
<td>34</td>
<td>41.35 (1.40)</td>
<td>5.9</td>
<td>-0.14 [-0.75, 0.47]</td>
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<tr>
<td></td>
<td>Birch</td>
<td>45</td>
<td>42.07 (1.22)</td>
<td>23</td>
<td>42.32 (1.11)</td>
<td>6.5</td>
<td>-0.25 [0.83, 0.33]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Makrides 1999</td>
<td>49</td>
<td>41.88 (1.22)</td>
<td>22</td>
<td>41.55 (1.11)</td>
<td>8.6</td>
<td>0.33 [0.25, 0.51]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lapillone</td>
<td>12</td>
<td>41.20 (1.20)</td>
<td>12</td>
<td>42.06 (1.03)</td>
<td>1.5</td>
<td>-1.40 [-2.62, -0.18]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Makrides 1995</td>
<td>13</td>
<td>41.69 (1.15)</td>
<td>19</td>
<td>42.16 (0.89)</td>
<td>2.5</td>
<td>-0.47 [-1.40, 0.49]</td>
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<td></td>
</tr>
<tr>
<td></td>
<td><strong>Total (95% CI)</strong></td>
<td><strong>378</strong></td>
<td><strong>20.0</strong></td>
<td><strong>17.0</strong></td>
<td><strong>0.13 (-0.01, 0.27)</strong></td>
<td><strong>100.0</strong></td>
<td><strong>0.01 [-0.14, 0.16]</strong></td>
<td><strong>0.9</strong></td>
<td></td>
</tr>
</tbody>
</table>

| 12 mo      | Carlson 1996 | 7 | 46.50 (0.77) | 9 | 46.34 (1.36) | 2.5 | 0.29 [0.39, 1.45] |
|            | Auestad 2001 | 119 | 46.54 (1.32) | 46 | 46.63 (1.20) | 20.3 | 0.01 [0.40, 0.42] |
|            | Morris    | 54 | 46.49 (1.29) | 54 | 47.03 (1.58) | 11.0 | -0.51 [0.07, 0.05] |
|            | Auestad 1997 | 89 | 45.62 (1.21) | 45 | 45.79 (1.25) | 17.7 | -0.18 [0.63, 0.27] |
|            | Carlson 1999 | 186 | 46.70 (1.40) | 68 | 46.89 (1.39) | 30.2 | -0.19 [0.44, 0.24] |
|            | Birch    | 39 | 46.81 (1.74) | 21 | 47.01 (1.25) | 8.0 | -0.40 [0.19, 0.30] |
|            | Makrides 1998 | 46 | 47.17 (1.37) | 21 | 46.62 (1.23) | 8.0 | 0.22 [0.44, 0.37] |
|            | Makrides 1995 | 12 | 46.31 (1.40) | 10 | 46.87 (1.05) | 4.1 | -0.56 [-1.49, 0.37] |
|            | **Total (95% CI)** | **551** | **314** | **100.0** | **0.14 [0.33, 0.05]** | **0.15** |

n-3 LCPUFA intervention only

<table>
<thead>
<tr>
<th>Time point</th>
<th>Study</th>
<th>LCPUFA n</th>
<th>mean (SD)</th>
<th>Control n</th>
<th>mean (SD)</th>
<th>WMD (95% CI Fixed)</th>
<th>Weight (%)</th>
<th>WMD (95% CI Fixed)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mo</td>
<td>Innis</td>
<td>68</td>
<td>41.52 (1.20)</td>
<td>36</td>
<td>41.46 (1.43)</td>
<td>21.0</td>
<td>0.12 [0.43, 0.67]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Auestad 1997</td>
<td>43</td>
<td>41.77 (1.10)</td>
<td>45</td>
<td>41.86 (1.06)</td>
<td>31.7</td>
<td>-0.09 [-0.54, 0.35]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Birch</td>
<td>22</td>
<td>42.00 (1.02)</td>
<td>20</td>
<td>42.32 (1.11)</td>
<td>18.0</td>
<td>-0.32 [0.99, 0.37]</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Lapillone</td>
<td>12</td>
<td>41.32 (1.20)</td>
<td>12</td>
<td>42.56 (1.04)</td>
<td>4.3</td>
<td>-1.40 [-2.62, -0.18]</td>
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</tr>
<tr>
<td></td>
<td>Makrides 1998</td>
<td>25</td>
<td>41.81 (1.63)</td>
<td>22</td>
<td>41.55 (1.11)</td>
<td>18.6</td>
<td>0.26 [0.33, 0.60]</td>
<td></td>
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<tr>
<td></td>
<td>Makrides 1995</td>
<td>13</td>
<td>41.89 (1.70)</td>
<td>18</td>
<td>42.16 (1.27)</td>
<td>5.5</td>
<td>-0.47 [1.56, 0.62]</td>
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</tr>
<tr>
<td></td>
<td><strong>Total (95% CI)</strong></td>
<td><strong>183</strong></td>
<td><strong>157</strong></td>
<td><strong>100.0</strong></td>
<td><strong>-0.10 [0.35, 0.16]</strong></td>
<td><strong>0.4</strong></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

| 12 mo      | Auestad 1997 | 43 | 46.67 (1.16) | 45 | 46.70 (1.25) | 44.8 | -0.03 [-0.54, 0.48] |
|            | * Birch    | 20 | 46.75 (1.19) | 21 | 47.01 (1.25) | 20.5 | -0.29 [-0.61, 0.04] |
|            | Makrides 1999 | 24 | 46.75 (1.08) | 21 | 46.95 (1.23) | 25.1 | -0.20 [0.88, 0.47] |
|            | * Makrides 1995 | 12 | 46.31 (1.72) | 18 | 46.87 (1.06) | 9.8 | -0.56 [-1.85, 0.53] |
|            | **Total (95% CI)** | **99** | **105** | **100.0** | **-0.17 [0.51, 0.17]** | **0.3** |

FIGURE 4. Meta-analysis forest plots for infant head circumference for all trials and for the subgroups intervening with only n-3 long-chain polyunsaturated fatty acid (LCPUFA). Trials are displayed according to increasing dose of n-3 LCPUFA, regardless of the presence of n-6 LCPUFA. Trials marked with an asterisk (*) had ceased dietary interventions at the indicated assessment. WMD, weighted mean difference.
associated with dietary n−3 LCPUFA supplementation in the absence of AA according to 6 trials. The subgroup analysis of those trials had dietary interventions that ranged in n−3 LCPUFA concentration from 0.1% to 1.0% total fatty acids, although most infants consumed formulas with 0.3–0.45% n−3 LCPUFA as total fatty acids (5, 7, 20, 27, 30). Despite the limited data there was no suggestion of an n−3 LCPUFA dose effect on any indicator of growth. All infants consumed the test formulas for at least 4 mo, and trials showed a mean reduction in plasma AA of ≈25% compared with control infants. Because growth is the main criteria used to assess nutritional health and well-being of infants, the fact that formulas supplemented with only n−3 LCPUFA are capable of supporting adequate infant growth, despite reductions in AA status, indicates that such formulas are nutritionally adequate. This finding is of considerable relevance because sources of AA for use in infant formulas are expensive and may add to the cost of infant formulas.

The often antagonistic actions of n−3 and n−6 LCPUFAs in a variety of metabolic systems has caused a reluctance on the part of some experts to endorse the supplementation of infant formula with n−3 LCPUFA without the addition of AA. DHA and AA are never absent from human milk. Although the concentration of AA in breast milk depends to some extent on the maternal diet, the amount of DHA in breast milk is almost entirely driven by dietary DHA. Claims of an optimum AA-to-DHA ratio of 2:1 have been based on amounts seen in the breast milk of some mothers in industrialized Western countries who consume diets low in n−3 fats but rich in n−6 fats (United States, Australia). It must be emphasized that the AA:DHA of human milk from women in other countries (and even American and Australian women who include fish in their diets) varies widely, and many Asian mothers have AA:DHA of 0.4:1, whereas the milk of many European mothers is closer to 1:1 (45, 46). Thus, the AA:DHA of 2:1 seen in some human milk, and suggested to be optimal, seems to only be part of a continuum that is diet driven and may have little relevance to the infant in relation to growth.

Subgroup analyses that investigate the effect of formula LCPUFA supplementation with either phospholipid or triacylglycerol sources also indicated little effect on infant growth. Although subgroup analyses are important, these data need to be interpreted with caution because of the comparatively smaller sample sizes and the increased number of comparisons. With this caveat in mind, our meta-analysis results are consistent with metabolic and biochemical studies that show little difference in the absorption of DHA and AA from both phospholipid and triacylglycerol sources (47) and that infant plasma and erythrocyte concentrations of DHA and AA depend on the dose of dietary supplementation rather than the source of supplementation (27, 32).

A decade after the publication of the first recommendations for LCPUFA supplementation of infant formulas (48–50) and the first published randomized trials of LCPUFA interventions, we have a wealth of quality information about growth and development. However, the impetus to add LCPUFAs to infant formula has been to improve the developmental outcome of infants, and this has resulted in the growth data from many trials often being inadequately reported, even though there has been some controversy about the effect of dietary LCPUFAs on growth. Our systematic review has clearly shown that LCPUFA supplementation of infant formulas for term infants does not affect growth and in this regard is safe. This review has attempted to exclude the possibility of publication bias by seeking details of growth data that were unpublished or not published in full. The positive response from both the investigators and the industry has allowed the completion of a comprehensive review in which there can be a high level of confidence.

MM and RAG designed the study and were responsible for coordinating funding. MM and RAG supervised KR and TU, who assisted with data abstraction and data analysis. MM and RAG wrote the paper with contributions from coauthors. MM and RAG have collaborated with the formula industry on clinical trials related to LCPUFA interventions, but the formula industry and the funding agencies for this project had no role in its design, analysis, and interpretation. All data were managed and analyzed in Adelaide, Australia. The authors had no associated financial interests. The International LCPUFA Investigators donated unpublished data and commented on the interim analyses and the final draft of the manuscript.

The International LCPUFA Investigators include Carlo Agostoni, Department of Pediatrics, The San Paolo Biomedical Institute, University of Milan, Milan, Italy; MaryAnn Aynn, Ross Products Division, Abbott Laboratories, Columbus, OH; Eileen Birch, Retina Foundation of the Southwest, Dallas, TX; Susan E Carlson, University of Kansas Medical Center, Kansas City, KS; Tamas Decsi, Department of Pediatrics, University Medical School of Pécs, Pécs, Hungary; Deborah Diersen-Schade, Mead Johnson Nutritional, Evansville, IN; William Goldberg, Wyeth Nutritional International, Philadelphia, PA; JS Forsyth, Department of Child Health, University of Dundee, Dundee, United Kingdom; Robert Hall, The Children’s Mercy Hospital, University of Missouri, Kansas City, MO; Cheryl L Harris, Mead Johnson Nutritional, Evansville, IN; Dennis Hoffman, Retina Foundation of the Southwest, Dallas, TX; Sheila M Imms, British Columbia Research Institute for Children’s and Women’s Health, University of British Columbia, Vancouver, Canada; Berthold Koletzko, Dr von Hauner Children’s Hospital, University of Munich, Munich, Germany; Alexandre Lapillonne, Rene Descartes University, Paris, France, and Children’s Nutrition Research Center, Baylor College of Medicine, Houston, TX; Kimberly L Merkel, Mead Johnson Nutritional, Evansville, IN; Michael Montalto, Ross Products Division, Abbott Laboratories, Columbus, OH; James Moorcraft, Royal Glamorgan Hospital, Llantrisant, United Kingdom; Geraint Morris, Singleton Hospital, Swansea, United Kingdom; Ricardo Uauy, Institute of Nutrition and Food Technology (INTA), University of Chile, Santiago, Chile; John CK Wells, Nutricia Baby Food Division, Trowbridge, United Kingdom; and Peter Willatts, Department of Psychology, University of Dundee, Dundee, United Kingdom.

REFERENCES


Longitudinal changes in energy expenditure in girls from late childhood through midadolescence

Jennifer L Spadano, Linda G Bandini, Aviva Must, Gerard E Dallal, and William H Dietz

ABSTRACT

Background: Longitudinal data on energy expenditure in children and adolescents are scarce.

Objective: The purpose of this study was to examine changes in energy expenditure and physical activity in girls from late childhood through midadolescence.

Design: We measured total energy expenditure (TEE) by doubly labeled water, resting metabolic rate (RMR) by indirect calorimetry, body composition by \(^{18}\)O dilution, and time spent in activity by an activity diary in 28 initially nonobese girls at \(\approx 10, \approx 12,\) and \(\approx 15\) y of age. Changes with age in TEE, RMR, and activity energy expenditure (AEE), both in absolute terms and in adjusted analyses, and in physical activity level (PAL) and time spent sleeping, being sedentary, and in moderate and vigorous activity were evaluated by mixed-model repeated-measures analyses.

Results: Absolute TEE and AEE increased significantly from age 10 to age 15 y \((P < 0.0001\) for both). Absolute RMR at ages 12 and 15 y did not differ significantly, despite significant increases in fat-free mass and fat mass between the visits. PAL was significantly higher \((P < 0.0001)\) at age 15 y than at age 10 or 12 y, whereas time spent being sedentary increased significantly from age 10 to age 15 y \((P < 0.001),\) and AEE adjusted for fat-free mass appeared to decrease over the same interval.


KEY WORDS Energy expenditure, resting metabolic rate, physical activity, parental overweight, puberty, adolescents, female, obesity

INTRODUCTION

Most data on energy expenditure (EE) in children and adolescents are cross-sectional in nature. Among the few longitudinal EE studies that have been conducted in children (1–6), only one study (6) has, to our knowledge, published data on changes in total EE (TEE), resting metabolic rate (RMR), and activity EE (AEE) during adolescence. Because it is believed to be a critical period in the development of obesity (7), adolescence is an important time in which to study changes in the components of EE. Such study is particularly important for females because obesity in adolescence is more likely to persist into adulthood in girls than in boys (8).

Obesity results from a chronic state of positive energy balance, in which energy intake exceeds EE. A decline in the most variable component of EE, physical activity (9), may play a role in the increasing prevalence of childhood overweight (10). Longitudinal (11) and cross-sectional (12) questionnaire data have shown a decline in leisure time and vigorous physical activity, respectively, during adolescence in females. Cross-sectional accelerometry data from children in grades 1–12 showed an inverse relation between school grade and the number of minutes per day of moderate to vigorous physical activity (13). Among the 3 published studies with longitudinal measures of physical activity based on EE, 1 study presented data on changes in AEE adjusted for race and obesity status rather than changes in absolute AEE or AEE adjusted for weight or body composition (6). The other 2 studies followed children from age 5 y to age 10 y.

Although AEE directly reflects the energy spent in activity, the energy cost of many activities is influenced by body weight (14–16). Consequently, absolute AEE is not the most appropriate indicator of relative physical activity. Several different approaches have been advocated to correct AEE for differences in body size and composition (16–18). Physical activity level (PAL), AEE per kg of fat-free mass (FFM) or per kg of body weight, and AEE adjusted for FFM or weight in statistical models have all been used.

The purpose of the current study was to examine in 28 females the changes that occur from late childhood to midadolescence in TEE, RMR, and AEE, and in physical activity as assessed by AEE adjusted for FFM, PAL, and time spent in activity as recorded in an activity diary. Although the relatively small size of our study sample means that any findings should be evaluated
cautiously and explored further in larger longitudinal studies, these data provide a rare opportunity to examine possible changes in EE during adolescence.

SUBJECTS AND METHODS

Between September 1990 and June 1993, 196 girls aged 8–12 y were enrolled in the Massachusetts Institute of Technology (MIT) Growth and Development Study, a prospective cohort study with annual follow-up visits from study entry until 4 y after menarche. Criteria for enrollment were premenarcheal status and a triceps skinfold thickness <85th percentile for age and sex (19). Girls were recruited from the Cambridge and Somerville (Massachusetts) public school systems and the MIT summer day camp; other recruits were friends and siblings of enrollees. All subjects were initially healthy and were not taking any medications known to affect body composition or metabolic rate.

Subjects in the current study were a subgroup (EE subcohort) of the MIT Growth and Development Study. Girls who enrolled in the MIT Growth and Development Study during year 2 or 3 of recruitment and were ≈10 y of age at study entry were asked to participate in a substudy designed to examine longitudinal changes in EE; 28 girls from different families agreed to participate.

Measurements of TEE by doubly labeled water, RMR by indirect calorimetry, body composition by total body water (TBW), and time spent in activity as recorded in an activity diary were taken at the baseline (year 0), year 2, and year 5 visits when the girls were ≈10, ≈12, and ≈15 y of age, respectively. The year 2 and year 5 visits were scheduled with ± 1 mo of the 2nd and 5th anniversary of the girl’s baseline visit, respectively. All 3 study visits were conducted during the school year. All 28 girls had a year 2 visit, and 24 of the 28 girls had a year 5 visit. Of the 4 girls missing data at year 5, 1 had moved out of the country, 1 dropped out of the study, and the remaining 2 could not schedule a visit before the end of the school year because of weekend extracurricular activities.

As part of the larger MIT Growth and Development Study cohort, the girls also had a 4th measure of RMR and TBW performed at their study completion visit, scheduled for 4 y (± 1 mo) after menarche. Twenty-three of the 28 girls came in for this final visit. However, the study completion visit coincided with the year 5 visit for 1 girl and preceded the year 5 visit for another girl; only data from the year 5 visit of these 2 girls were included in these analyses. In addition, 1 girl was missing TBW data at study completion. Consequently, a 4th measure of RMR and TBW was available for only 20 of the 28 girls. Of the 5 girls without this study completion visit, 2 dropped out of the study before their year 5 visit, 2 dropped out of the study between their year 5 visit and their scheduled study completion visit, and 1 had moved and could not be located for her study completion visit (referred to below as the visit 4 y after menarche).

Written informed consent was obtained from both the subject and a parent or legal guardian (when subject was <18 y old) at each study visit. The study was approved by both the Committee on the Use of Humans as Experimental Subjects at MIT (Cambridge, MA) and the Institutional Review Board at the Tufts-New England Medical Center (Boston, MA).

Total energy expenditure and body composition

For all 4 study visits, subjects were admitted to the General Clinical Research Center (GCRC) at MIT in the late afternoon for an overnight stay. On the girl’s arrival, the study physician obtained a medical history and performed a brief medical examination to assess the girl’s health. At the baseline, year 2, and year 5 visits, a baseline urine sample was collected, and an overnight fast was initiated approximately 1 h before the administration of D2H18O. In the evening, between 1900 and 2000, a dose of 0.25 g H218O and 0.1–0.12 g H216O per kg of estimated TBW was administered to the study subject. Urine was collected until 0600 the next morning to determine urinary losses of isotope. The second urine void of the morning was used to measure 18O and 2H enrichment above the baseline values. This sample was used to determine TBW and served as the initial time point of the EE period (initial sample). Subjects returned to the GCRC as outpatients 2 wk after admission. At this time, the 2nd urine void of the day (endpoint sample) was collected to complete the EE period. Isotopic enrichments of the urine samples were measured on a Hydras Gas Isotope Ratio Mass Spectrometer (PDZ Europa Ltd, Northwich, United Kingdom) at the mass spectrometry laboratory at the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University (Boston). Criteria for acceptance values were replicate-measures SEs of 0.35 for 18O and 1.5 for 2H.

We used a modification (20) of the equation of Lifson and McClintock (21) to calculate the mean daily rate of carbon dioxide production (mol CO2/d), as follows:

\[ r_{CO_2} = \frac{(N/2.078)(1.01 k_o - 1.04 k_f) - 0.0246r_{GI}}{2} \]

where N is TBW in mol, \( k_o \) is the 18O elimination rate, \( k_f \) is the 2H elimination rate, and \( r_{GI} \) is the estimated rate for isotopically fractionated water loss, equal to 1.05N(1.01 k_o – 1.04 k_f).

The elimination rates of the 18O and 2H isotopes were calculated according to the 2-point method using the difference in the atom percent excess (APE) of the initial (i) and endpoint (f) samples and the time between their urine collections, as follows:

\[ k = \frac{(\ln APE_i - \ln APE_f)}{\text{time}} \]

where APE is the isotopic enrichment of a sample relative to that of the predose (baseline) sample. TEE was calculated by using Weir’s equation (22). Oxygen consumption was obtained by dividing rCO2 by the food quotient (23) derived from a 7-d food diary that subjects collected during the 2nd wk of the EE period, as described elsewhere (24). For the 2 girls at baseline and the 3 girls at year 5 who lacked valid food diaries, we used the average food quotient for the entire prospective cohort at baseline (as reported previously in 25) and the average food quotient for the subcohort at year 5, respectively.

At the baseline visit, at years 2 and 5, and 4 y after menarche, body composition was estimated from TBW by using 18O dilution space. The dose of 18O administered at the visit 4 y after menarche was 0.07 g H218O/kg estimated TBW, which followed the doubly labeled water protocol described earlier from the collection of the baseline urine sample to the collection of the 2nd urine void of the morning after the overnight stay. The oxygen dilution space was calculated according to the method of Halliday and Miller (26) and was assumed to be 1% higher than TBW. FFM was estimated from TBW by assuming a hydration constant of 0.73. Fat mass (FM) was calculated as the difference between body weight and FFM, and percentage body fat was calculated by dividing FM by body weight and multiplying by 100.
Resting metabolic rate

RMR was measured in the morning by using an indirect calorimeter with customized software and fitted with a ventilated hood, as described previously (25, 27). Each subject fasted overnight for a minimum of 12 h and engaged in minimal activity before the determination of metabolic rate. A 30-min rest and a 5-min equilibration period preceded the 30-min measurement period. On the morning of each measurement, the linearity of the gas analyzers was confirmed by calibrating the analyzers against 2 standard gases and checking the concentration of a third standard gas. In addition, the study technician checked the calibration of the entire system before each scheduled visit by pushing known amounts of a standard gas through the hood at a constant rate with a 3-L calibrated syringe (Warren E Collins Co, Braintree, MA). RMR was calculated from measures of oxygen consumption and carbon dioxide production according to modified Weir’s equation (22). At the baseline, year 2, and year 5 visits, RMR was calculated from measures of oxygen consumption and carbon dioxide production according to modified Weir’s equation (22). At the baseline, year 2, and year 5 visits, RMR was measured on the morning after subjects were admitted to the GCRC and ∼2 wk later when they returned to end their EE period. The average of the 2 RMR values at each time point was used. The intraclass correlation of the 2 RMR measures at baseline for the entire prospective cohort was 0.96, which indicated that the measurements were highly reproducible (25). Consequently, RMR at the visit 4 y after menarche was based on a single measurement made the morning after the overnight stay.

Measures of physical activity

AEE was calculated as \((0.9 \times \text{TEE}) - \text{RMR}\), assuming that 10% of TEE is food-induced thermogenesis (9). PAL was calculated as TEE/RMR.

Subjects kept an activity diary during the 1st week of the EE period for the baseline, year 2, and year 5 visits. The diary was set up as a grid with the rows representing each hour of the day and the columns indicating sleep, sit, stand, walk, and play. The girls documented when they awoke and when they went to sleep. For each 1-h time block in between, the girls were instructed to place an X in the column(s) that best described their activity during that hour, recording up to 2 activities/h. In accordance with the baseline coding protocol for the larger prospective cohort, the time attributed to each activity within a given hour was based on the number of X markings within a row (eg, 1.0 h for 1 activity checked, 0.5 h each for 2 activities checked). When a 1-h time block between awakening in the morning and going to sleep at night was left blank, the activities sit, stand, walk, and play were each assigned 0.25 h. The time spent sleeping, sitting, standing, walking, and playing for each day was then calculated. The average of the 2 RMR values at each time point was used. The intraclass correlation of the 2 RMR measures at baseline for the entire prospective cohort was 0.96, which indicated that the measurements were highly reproducible (25). Consequently, RMR at the visit 4 y after menarche was based on a single measurement made the morning after the overnight stay.

Other variables

Weight was measured in the morning, while subjects were in a fasted state, by using a digital scale (Seca, Hamburg, Germany) that was accurate to 0.1 kg. Height (without shoes) was also measured at this time by using a wall-mounted stadiometer that was accurate to 0.1 cm. We used the Centers for Disease Control and Prevention 2000 growth charts (28) to calculate percentiles of body mass index (BMI; in kg/m²) for age based on each girl’s measured height and weight at each visit. Race or ethnicity (ie, white, black, Hispanic, Asian, and other) was based on self-report on a questionnaire completed at study entry; 19 girls (68%) identified themselves as white, 5 as black, 1 as Hispanic, 2 as Asian, and 1 as other. For all analyses, race or ethnicity was further categorized into a dichotomous variable (black or non-black).

Tanner staging (29) of breast development was assessed by either a study physician or a female coinvestigator at each visit up until menarche. The girls were instructed to call the study personnel when they had their first period. Some girls reported their date of menarche during one of their annual follow-up visits. At these visits, girls were asked if they had started their period during the preceding year; if the answer was yes, the girl was asked to recall the date.

Early in the study, the heights and weights of the biological parents of each girl were collected either by self-report or by measurements taken at MIT (in normal clothing, without shoes). Only 3 girls were missing data necessary to classify parental weight status. Among the remaining 25 girls, only 1 had self-reported rather than measured data. Parental overweight was defined as a BMI ≥ 25 (30). Girls were classified as having 2 normal-weight biological parents (NWP) or at least 1 overweight biological parent (OWP).

Statistical analysis

All statistical analyses were performed by using SAS software (version 8.1; SAS Institute, Cary, NC). Mean (±SD) age, height, weight, BMI-for-age percentile, percentage body fat, FM, and FFM were calculated for each visit. Mixed-model repeated-measures analyses (using PROC MIXED in SAS) were performed to evaluate the changes with age in TEE, RMR, and AEE both before and after adjustment for key covariates; covariates considered for inclusion in each model were race, parental overweight, pubertal status, FFM, and FM (for RMR only). Because of our earlier observation of a higher absolute RMR at menarche (± 6 mo) than at 4 y after menarche (31), we assessed the influence of puberty on EE, with pubertal status expressed in terms of the timing of each visit relative to menarche. For each visit, a girl’s pubertal status was retrospectively classified as >1 y before menarche, 1 y before menarche to 6 mo after menarche, or >6 mo after menarche. Because the exact timing and duration of the proposed elevation in RMR are unclear, we evaluated 2 additional pubertal status variables: 1) >1 y before menarche, within 1 y of menarche, and >1 y after menarche and 2) >1.5 y before menarche, 1.5 y before menarche to 6 mo after menarche, and > 6 mo after menarche. The last of the 3 proposed pubertal status variables performed better overall in cross-sectional analyses as assessed by a comparison of each model’s adjusted \( r^2 \) value and the \( P \) value of its pubertal status variable. Hence, we selected this variable for consideration in the mixed models. We
also alternatively evaluated time until menarche as a continuous, time-varying covariate.

Because RMR adjusted for body composition appears to be lower in black than in white girls (2, 25, 32–34), we evaluated a race \times visit interaction term to assess any racial differences in the changes that occur in TEE and its components with age. In addition, we tested a parental overweight \times visit interaction term in all adjusted analyses because we previously observed in another subgroup of girls a significant parental overweight \times visit interaction in RMR evaluated before menarche (baseline), at menarche, and 4 y after menarche (31). We also determined the significance of changes with age in absolute FFM, FM, PAL, and time spent sleeping, being sedentary, and in moderate and vigorous activity. Time was modeled as a categorical variable (ie, visit) in all analyses to allow comparisons between the visits. Final models in which time was included as either a categorical or continuous variable were compared by using the maximum log-likelihood ratio test to determine whether the data were consistent with linearity. The covariance structure was retested after each change to a given model by using the log-likelihood ratio test for nested models and Akaike’s information criterion (35) for nonnested models. Tukey’s honestly significant differences were used to evaluate the differences across age. Results were considered significant if the observed \( P \) value was < 0.05.

RESULTS

At baseline, 19 (68%) of the 28 girls were classified as Tanner stage 1, and the remaining 9 girls were pubertal, although nonmenarcheal. Mean age, height, weight, BMI-for-age percentile, percentage body fat, FM, and FFM at each visit are shown in Table 1. Significant increases in both FFM and FM were observed from \( \approx 10 \) to \( \approx 12 \) to \( \approx 15 \) y of age; FFM increased from 25.3 to 32.3 to 42.0 kg, and FM increased from 8.4 to 13.1 to 16.4 kg, respectively (\( P < 0.0001 \) for FFM and \( P < 0.001 \) for FM for the differences between visits). Between the year 5 visit and the visit 4 y after menarche (\( \bar{x} \) interval: 1.9 ± 0.8 y), mean FFM increased significantly (\( P < 0.01 \), by \( \approx 1.4 \) kg, and mean FM appeared to increase by \( \approx 0.8 \) kg (\( P = 0.32 \)). Mean (±SD) age at menarche for this subcohort was 12.5 ± 0.9 y.

Of the 25 girls with data on parental weight status, 10 had 2 NWP (NWP girls), and 15 had at least 1 OWP (OWP girls). Among the 3 girls missing data on parental overweight, 1 was white and 2 were black. One NWP girl who dropped out of the study before she experienced menarche was missing data on menarcheal age and was not included in models considering pubertal status.

Mean absolute TEE increased significantly at each age, rising from 8176 to 9355 to 10 364 kJ/d at \( \approx 10, \approx 12, \) and \( \approx 15 \) y, respectively (Figure 1). Overall, FFM (\( P < 0.0001 \); direct association), race (\( P < 0.0001 \); lower in blacks), and pubertal status (\( P < 0.001 \); inverse association) were significant predictors of TEE, and therefore they were included in the final model. In addition, the parental overweight \times visit interaction term was significant (\( P < 0.001 \)). The adjusted means of TEE for each parental weight group at each visit are shown in Figure 2. Although TEE adjusted for FFM, race, and pubertal status appeared to increase with age in the NWP girls and to decrease with age in the OWP girls, within each parental weight group, the differences in adjusted TEE between the visits were not significant. The results did not differ significantly between the models in which time was a categorical or continuous variable.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n = 28)</th>
<th>Year 2 (n = 28)</th>
<th>Year 5 (n = 24)</th>
<th>Menarche +4y (n = 21)</th>
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</thead>
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<tr>
<td>Age (y)</td>
<td>9.9 ± 0.4a</td>
<td>11.9 ± 0.4b</td>
<td>14.8 ± 0.4c</td>
<td>16.6 ± 0.9d</td>
</tr>
<tr>
<td>Time relative to menarche (y)</td>
<td>−2.7 ± 0.9a</td>
<td>−0.7 ± 0.9b</td>
<td>2.3 ± 1.0c</td>
<td>4.1 ± 0.1d</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>141.1 ± 5.7a</td>
<td>154.3 ± 6.6b</td>
<td>164.5 ± 6.3c</td>
<td>166.9 ± 5.9d</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>33.7 ± 4.7a</td>
<td>45.3 ± 7.1b</td>
<td>58.4 ± 6.9c</td>
<td>60.7 ± 7.3d</td>
</tr>
<tr>
<td>BMI-for-age percentile</td>
<td>49.6 ± 26.0a</td>
<td>56.6 ± 28.4b</td>
<td>64.9 ± 22.6b</td>
<td>58.4 ± 24.2ab</td>
</tr>
<tr>
<td>Percentage body fat (%)</td>
<td>24.6 ± 4.8a</td>
<td>28.2 ± 6.9b</td>
<td>27.7 ± 5.0b</td>
<td>28.0 ± 5.2b</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>8.4 ± 2.5a</td>
<td>13.1 ± 4.7b</td>
<td>16.4 ± 4.2c</td>
<td>17.2 ± 4.6c</td>
</tr>
<tr>
<td>Fat-free mass (kg)</td>
<td>25.3 ± 3.0a</td>
<td>32.3 ± 4.4b</td>
<td>42.0 ± 4.4c</td>
<td>43.4 ± 4.4ad</td>
</tr>
</tbody>
</table>

\(^{1}\) All values are \( \bar{x} \) ± SD. Values in the same row with different superscript letters are significantly different, \( P < 0.05 \) (unadjusted mixed-model repeated-measures analyses with Tukey’s honestly significant differences).

\(^{2}\) \( n = 20 \) for percentage body fat, fat mass, and fat-free mass.

![Figure 1](image-url)  
**FIGURE 1.** Absolute total energy expenditure of 28 girls at baseline, year 2, and year 5, corresponding to \( \approx 10, \approx 12, \) and \( \approx 15 \) y of age, respectively. Significance was assessed by mixed-model repeated-measures analysis with Tukey’s honestly significant differences used in the comparisons of the means. Bars with different superscript letters are significantly different from one another (\( P < 0.0001 \)). Error bars represent the 95% CI (1). \( n = 24 \) at year 5.
FIGURE 2. Adjusted $\bar{X}$ total energy expenditure of 9 girls with 2 normal-weight parents (NWP) and 15 girls with ≥1 overweight parent (OWP) at baseline, year 2, and year 5, corresponding to $\approx$10, $\approx$12, and $\approx$15 y of age, respectively. Adjusted means of a significant parental overweight × visit interaction term are presented from a mixed-model repeated-measures analysis containing fat-free mass, race (black or nonblack), pubertal status (>1.5 y before menarche, 1.5 y before menarche to 6 mo after menarche, or 6 mo after menarche), parental overweight (NWP or OWP), and visit ($P < 0.001$). Tukey’s honestly significant differences were used in the comparisons of the means. Bars with different superscript letters represent the 95% CI ($n = 13$ in the OWP group at year 5).

Mean absolute RMR was significantly lower at age $\approx$10 y (5226 kJ/d) than at age $\approx$12 y (5929 kJ/d), age $\approx$15 y (5853 kJ/d), and at 4 y after menarche ($\bar{x}$ age: 16.6 y; 5820 kJ/d) (Figure 3). Although the differences in mean RMR between the visits at year 2, year 5, and 4 y after menarche were not significant, the observation that mean absolute RMR at year 2 was 76 kJ/d higher than at year 5, despite the average accumulation of an additional 9.7 kg FFM and 3.3 kg FM between the 2 visits, is unexpected. Overall, FFM ($P < 0.0001$; direct association) and race ($P < 0.01$; lower in blacks) were significant predictors of RMR, whereas FM was marginally significant ($P = 0.05$); all 3 variables were included in the final model. In addition, the parental overweight × visit interaction term was significant ($P < 0.001$). The adjusted means of RMR for each parental weight group at each visit are shown in Figure 4. Among the NWP girls, mean adjusted RMR was 5766, 5983, 5054, and 4674 kJ/d at $\approx$10, $\approx$12, $\approx$15, and 16.6 ($\bar{x}$) y of age, respectively. Mean adjusted RMR was significantly higher at year 2 than at year 5 ($P < 0.001$) and at 4 y after menarche ($P < 0.0001$). In the OWP girls, adjusted RMR was significantly higher at baseline and year 2 than at year 5 and at 4 y after menarche ($P < 0.0001$ for all). Error bars represent the 95% CI ($n = 9$ in the NWP group and $n = 13$ in the OWP group at year 5; $n = 8$ in the NWP group and $n = 10$ in the OWP group at 4 y after menarche).

FIGURE 3. Absolute resting metabolic rate in 28 girls at baseline, year 2, year 5, and 4 y after menarche, corresponding to $\approx$10, $\approx$12, $\approx$15, and (x) 16.6 y of age, respectively. Significance was assessed by mixed-model repeated-measures analysis with Tukey’s honestly significant differences used in the comparisons of the means. Bars with different superscript letters are significantly different from one another ($P < 0.0001$). Error bars represent the 95% CI (1). $n = 24$ at year 5; $n = 21$ at 4 y after menarche.

FIGURE 4. Adjusted resting metabolic rate in 10 girls with 2 normal-weight parents (NWP) and 15 girls with ≥1 overweight parent (OWP) at baseline, year 2, year 5, and 4 y after menarche, corresponding to $\approx$10, $\approx$12, $\approx$15, and (x) 16.6 y of age, respectively. Adjusted means of a significant parental overweight × visit interaction term are presented from a mixed-model repeated-measures analysis containing fat-free mass, fat mass, race (black or nonblack), parental overweight (NWP or OWP), and visit ($P < 0.001$). Tukey’s honestly significant differences were used in the comparisons of the means. In the NWP girls, adjusted RMR at baseline and year 2 were not significantly different. However, adjusted RMR was significantly higher at baseline than at 4 y after menarche ($P < 0.01$), and adjusted RMR was significantly higher at year 2 than at year 5 ($P < 0.001$) and at 4 y after menarche ($P < 0.0001$). In the OWP girls, adjusted RMR was significantly higher at baseline and year 2 than at year 5 and at 4 y after menarche ($P < 0.0001$ for all). Error bars represent the 95% CI ($n = 9$ in the NWP group and $n = 13$ in the OWP group at year 5; $n = 8$ in the NWP group and $n = 10$ in the OWP group at 4 y after menarche).

Absolute AEE increased significantly from 2134 to 2489 to 3502 kJ/d from $\approx$10 to $\approx$12 to $\approx$15 y of age, respectively ($P < 0.05$ for all) (Figure 5). Overall, FFM ($P < 0.0001$; direct association), race ($P = 0.04$; lower in blacks), and pubertal status ($P = 0.02$; inverse association) were significant predictors of AEE and therefore are included in the final model. Visit was not significant ($P = 0.51$) in the model that included pubertal status; mean adjusted AEE was 2548, 2402, and 2607 kJ/d at $\approx$10, $\approx$12, and $\approx$15 y of age. The pattern of change in adjusted AEE with age.
did not differ significantly by parental weight status. When pubertal status and race were removed from the model to allow a comparison of age-related changes in physical activity with PAL and time spent in activity (unadjusted results for each of the latter 2 outcome variables are presented below), mean AEE adjusted for FFM appeared to decline with age from 721 to 609 and to 573 kJ/d at ≈10, ≈12, and ≈15 y of age, respectively; however, only the difference between baseline and year 2 was significant ($P = 0.04$).

Absolute PAL was significantly higher ($P < 0.0001$) at ≈15 y of age (1.77) than at ≈10 and ≈12 y of age (both 1.57; Figure 6). The pattern of change in PAL across the 3 visits did not differ significantly between the NWP and OWP girls. Overall, FFM was a significant predictor of PAL ($\beta = 0.013, P = 0.004$), as was weight when evaluated in separate models ($\beta = 0.006, P = 0.04$).

Mean values by age for the time spent sleeping, being sedentary, and in moderate and vigorous activity, taken from the activity diary, are shown in Figure 7. On average, time spent sleeping declined significantly, from 10.8 h/d at ≈10 y of age to 9.7 h/d by ≈15 y of age ($P < 0.0001$). Sedentary time increased significantly, by ≈2 h/d, from ≈10 to ≈15 y of age ($P < 0.001$). Time spent in moderate and in vigorous activity appeared to decline over the same interval, although the differences were smaller and not significant. When the hours spent in moderate and in vigorous activity were summed to reflect nonsedentary time, the observed differences by age still were not significant. Mean nonsedentary time was 3.7, 3.3, and 3.0 h/d at ≈10, ≈12, and ≈15 y of age, respectively ($P = 0.15$ for time modeled as categorical and $P = 0.06$ for time modeled as continuous). For each of the activity diary variables, the results with categorical time (ie, visit) did not differ significantly from those with time modeled as a continuous variable.

**DISCUSSION**

Our study measured longitudinal changes in EE during adolescence, a period that is believed to be critical in the development of obesity. Longitudinal studies of EE are rare, particularly in adolescents, because of the high costs associated with repeated measures of EE and the challenge of retaining adolescents in longitudinal studies. Therefore, our data are unique. However,
our findings must be viewed cautiously because of the sample size and the complexity of our adjusted analyses.

Only a few longitudinal studies have published data on changes in TEE, RMR, or AEE (or all) in children (1–6, 31). One study was restricted to boys (4). Two other studies looked exclusively at changes in metabolic rate: Sun et al (2) found an inverse relation between Tanner stage and adjusted RMR, whereas in a previous study, we (31) found results consistent with the current study. In a study whose results were also consistent with the current findings, mean TEE adjusted for FFM in a study of 8 girls did not differ significantly at ~10.4 and ~12.8 y of age (3). Two-year follow-up data from the Baton Rouge Children’s Study on changes in TEE, RMR, and AEE adjusted for race and obesity status were presented (6). Changes in absolute AEE or EE adjusted for changes in body size or body composition were not reported. A study of Pima Indian children found that mean absolute TEE and AEE increased by 60% and 150%, respectively, between ages 5 and 10 y (5). In contrast, in a study by Goran et al (1), mean absolute TEE increased in 11 girls from age 5.5 to 6.5 y and then declined significantly, by a mean of 866 kJ/d, by age 9.5 y. This decline was attributed to a 50% reduction in AEE, which was hypothesized to be an energy-conserving mechanism in girls just before puberty (1). In the current study, however, we did not observe a decline in either absolute TEE or AEE with age; nor do our results suggest a decline in AEE just before puberty. Mean absolute AEE increased from 2276 to 2481 kJ/d from baseline to year 2 in the 16 girls who became pubertal during this period.

Except for our earlier publication on RMR (31), none of the aforementioned studies on longitudinal EE, to our knowledge, considered the potential influence of parental overweight. In the current study, we found that changes with age in adjusted RMR and TEE, but not AEE, differed according to parental weight status. These findings suggest that the observed influence of parental overweight on TEE is driven by genetic influences on RMR. However, because of the complexity of the adjusted TEE and RMR analyses in the small sample size in our study, these findings regarding parental overweight should be viewed as hypothesis-generating observations that require confirmation in other study populations.

We found that mean absolute RMR at ~12 y of age did not differ significantly from mean RMR at ~15 y of age, despite significant increases during the interim in both FFM, the major determinant of RMR (36), and FM, an independent contributor to RMR (25, 34, 37, 38). This observation is consistent with our earlier findings in 44 girls of a significantly higher mean absolute RMR at menarche (~6 mo) than at 4 y after menarche (31). Among the girls in the current study, one-third were within ~6 mo of menarche, and all but 2 were pubertal but nonmenarcheal; 7 girls were included in both studies. Therefore, the findings of the current study coupled with those published earlier (31) suggest that the observed elevation in RMR is not specific to menarche but most likely begins in midpuberty and persists through menarche. The lack of significance of the pubertal status variable in the adjusted RMR model may reflect the sparseness of RMR measures around menarche as well as the study’s limited power. Plausible mechanisms for the proposed elevation in RMR are discussed elsewhere (31).

We assessed age-related changes in physical activity by using PAL, AEE adjusted for FFM, and the time spent in activity as recorded in an activity diary. The observed increase in PAL suggests an increase in physical activity in midadolescence. In contrast, the changes with age in AEE adjusted for FFM and in sedentary time both suggest that the girls in our study became less active with age. Westerterp (39) showed that the fraction of the day spent in activities of moderate intensity significantly predicts PAL, whereas no relation was found between PAL and the time spent in high-intensity activity, presumably because of its relatively short duration. If this observation in adults is broadly applicable, then TEE and PAL likely are more influenced by the interaction between the relative proportions of time spent sleeping (a decrease) and being sedentary (an increase) might explain the increased PAL at age 15 y because there is a higher energy cost associated with being sedentary [metabolic equivalent (MET): 1.1–1.9 (40, 41)] than with sleeping (MET: ~0.9). In that scenario, however, one would expect adjusted AEE to rise along with PAL, but that is contrary to our findings.

In a meta-analysis of data from 17 doubly labeled water studies conducted in children, Hoos et al (42) attributed the age-related increases in PAL to increases in body weight. Their conclusion was based on their findings of a positive association between age and PAL and of no association between age and AEE/kg body wt. A similar conclusion was reached by Ekelund et al (18) on the basis of their cross-sectional findings that PAL and absolute AEE were significantly higher and that AEE/kg FFM and body movement, as measured with an accelerometer, were significantly lower in adolescents than in children. Our previous findings of a positive influence of body weight on the MET values of walking (43) indirectly support the notion that body weight influences PAL, because both PAL and MET share the assumption that dividing EE by RMR removes the influence of weight. In addition, in separate models estimated in the current study, both weight and FFM were significantly related to the changes in PAL with age.

In addition to the small sample size, several potential limitations of our study are noteworthy. First, because our sample was predominantly white and middle-class, the age-related changes we observed in TEE and physical activity may not be generalizable to other groups. Second, we assumed that the pattern of change in the various components of EE did not differ between black and nonblack girls. Although we found no race × visit interaction for adjusted TEE, AEE, or RMR, our sample provided limited power. Third, we obtained only a single measure of TEE at each age. A single measure may not represent habitual EE if the doubly labeled water measurement is performed during a relatively low or high period of activity (44). The within-subject variation in doubly labeled water measurements attributed to analytic and inherent biological variation is estimated at 8% (44). Fourth, we cannot rule out the possibility that qualitative changes in reporting accuracy may influence the age-related changes observed in time spent in activity. Children’s accuracy in self-reporting activity may improve with age (45) or, alternatively, may decline with age if children no longer seek help from their parents or become less enthusiastic and hence less diligent in their recording as adolescents.

In conclusion, our data show a discrepancy in age-related changes in physical activity between PAL and both AEE adjusted for FFM and time spent in activity. PAL may be influenced by body weight. Until the most valid measure of age-related changes in physical activity is identified, the role that physical activity plays in the development of childhood obesity will remain uncertain.
The authors gratefully acknowledge the girls who participated in this study and the staff at the General Clinical Research Center at the Massachusetts Institute of Technology for their assistance.

In addition to making intellectual contributions to the manuscript, LGB and WHD designed the study and collected data, AM and GED provided statistical advice, and JLS collected data, performed the oxygen isotope analyses and the statistical analyses, and wrote the manuscript. None of the authors had any personal or financial conflicts of interest.

REFERENCES


I. INTRODUCTION

Homocysteine is a sulfur-containing amino acid formed from methionine during $\text{S-adenosylmethionine-dependent methylation}$ reactions. Further metabolism of this amino acid is dependent on several B vitamins. Vitamin $B_6$ is the cofactor of cystathionine $\beta$-synthase, the enzyme that irreversibly converts homocysteine to cystathionine. Folate in the 5-methyltetrahydrofolate form donates its methyl group to homocysteine by methionine synthase and requires vitamin $B_12$ as a cofactor. The enzyme methionine tetrahydrofolate reductase (MTHFR) reduces 5,10-methyltetrahydrofolate to 5-methyltetrahydrofolate. The $677C\rightarrow T$ polymorphism in the MTHFR gene decreases its enzyme activity (1).

Hyperhomocysteinemia, defined as a moderately elevated total homocysteine (tHcy) concentration, is an established independent risk factor for arterial vascular disease and venous thrombosis in adults (2–4). Data on this relation in childhood are rare, but Cardo et al (5, 6) and our group (7, 8) found that hyperhomocysteinemia is a risk factor for ischemic stroke in children. Koch et al (9) linked elevated tHcy to an increased risk of venous thrombosis in children. tHcy and its predictors have been studied more extensively in adult populations than in children. The tHcy concentration is influenced by several determinants, such as age; sex; plasma folate, vitamin $B_6$, vitamin $B_12$, and creatinine concentrations; and the use of hormones, vitamin supplementation, and anti-folate medications (10). The MTHFR $677C\rightarrow T$ polymorphism is the most prevalent genetic cause of hyperhomocysteinemia, particularly under conditions of impaired folate status (11). tHcy is elevated in pathologic conditions such as renal failure, thyroid dysfunction, and malignancy (12).

In the past decade, information on plasma tHcy in children has begun to emerge (13–36). Although age and sex were taken into account, those studies tended to target specific subgroups such as neonates (13–17), groups of smaller age ranges (18–25), or older children (26–31). Four studies included subjects whose age ranged from 0 to 19 y, but one of those studies measured only tHcy concentrations (32). Another study investigated the effect of MTHFR polymorphism on tHcy, but data on vitamin $B_12$ status were unavailable (33). In 2 other studies, the MTHFR genotyping was missing (34, 35). In most studies, a negative correlation of tHcy with plasma folate and with vitamin $B_12$ concentrations was found (13–15, 19, 20, 22–27, 29, 30). The correlation between tHcy and plasma folate was significantly stronger than that between tHcy and vitamin $B_12$; the correlation between tHcy and plasma folate also varied among the age groups. Data on the effect of MTHFR genotype on tHcy and the interference between vitamin status and MTHFR genotype were limited to one study of 127 children (36). Therefore, we designed a study to investigate...

1. From the Departments of Pediatrics (IMvB, LA, DO-vE, and HJB), Endocrinology (MdB), Epidemiology and Biostatistics (MdB), Obstetrics and Gynecology (CMGT), and Chemical Endocrinology (CMGT), Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands.
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3. Reprints not available. Address correspondence to IM van Beynum, Department of Pediatrics, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, Netherlands. E-mail: i.vanbeynum@cukz.umcn.nl. Received January 29, 2004. Accepted for publication January 11, 2005.
tHcy concentrations and their possible predictors in Dutch children over the whole range of childhood.

SUBJECTS AND METHODS

Subjects and data collection

Over a 3-mo period during 1997, 234 white children were recruited to participate in this study. All participants were aged 0–19 y. We included apparently healthy volunteers from secondary schools (aged 11-19 y) in the Nijmegen area. Children aged <11 y were recruited in the Pediatric Clinic of the University Hospital in Nijmegen. When blood was drawn for diagnostic or follow-up investigations, the children or their parents (or both) were asked to donate some blood in the same venipuncture session for use in this study. The exclusion criteria were overt liver, thyroid, and renal dysfunction; hormonal therapy; anti-folate medication; neoplastic disease; closure defects such as cleft lips and spina bifida; and occlusive arterial and venous disease. Additional information about medical history, use of medication, smoking behavior, and puberty features (in girls, the occurrence of menarche; in boys, the growth of beard hair) was obtained from medical records or was collected by written questionnaire.

Informed consent (sometimes written, sometimes oral) was obtained from all the children’s parents and from children who were old enough to provide it. The study protocol was approved by the local medical ethics committee.

Blood sampling and biochemical determination

Blood samples for total tHcy measurement were drawn by venipuncture into 3-mL evacuated tubes containing EDTA. In neonates, a capillary blood sample was obtained in 1-mL microtainers containing EDTA. The EDTA sample was immediately placed on ice and centrifuged within 4 h at 2000 × g for 10 min. The plasma was separated and stored at −20 °C until analysis. The remaining cells were stored at −20 °C and used for DNA isolation. If possible, a venous blood sample was taken into 5-mL heparin-containing evacuated tubes for plasma folate, vitamin B-12, and creatinine measurements. These plasma samples were also stored at −20 °C. The blood sample for tHcy measurement, which requires a smaller volume, had priority in the youngest children. From the 234 participants, 189 heparinized blood samples were obtained for plasma folate and vitamin B-12 measurement. For plasma creatinine measurement, 178 samples were obtained. For 220 samples, DNA isolation and genotyping were successful.

Plasma tHcy concentrations were measured by using an automated HPLC method with reverse-phase separation and fluorescence detection (Gilson 232–401 sample processor; Gilson, Middleton, WI; Spectra Physics 8800 solvent delivery system and Spectra Physics LC 304 fluorometer; Spectra Physics, San Jose, CA), as described by Fiskerstrand et al, with some modifications (37–39). Plasma folate and vitamin B-12 concentrations were measured by using the Dualcount Solid Phase Boil Radioassay (Diagnostic Products, Los Angeles, CA). The investigated mutation in the MTHFR gene is a C-to-T substitution at base pair 677 that alters an alanine to a valine residue. This mutation creates a Hinfl site, designated 677T, allowing for restriction site analysis. The prevalence of the C677T mutation was investigated by polymerase chain reaction in genomic DNA extracted from blood leukocytes, which was followed by restriction enzyme digestion with Hinfl and detection with the use of agarose gel electrophoresis (40).

Statistical analysis

We performed statistical analysis with SPSS software (version 11.5; SPSS Inc, Chicago, IL). The distributions of the plasma concentrations of tHcy, folate, and vitamin B-12 appeared to be skewed toward higher values. Logarithmic transformations were applied to normalize these distributions. Inverse transformations were performed to provide geometric means and 95% CIs. Because of the significant age dependency of tHcy, plasma folate, vitamin B-12, and creatinine concentrations, we tabulated these variables in 5 different age groups (ie, 0–1, 2–5, 6–10, 11–14, and 15–19 y); there was an equal number of subjects in each group for tHcy measurement. Males and females were tabulated separately, although the interaction between age and sex was not significant. The difference in tHcy concentration between males and females was expressed in a ratio of males to females, with 95% CIs, calculated in a linear regression model. Correlations were calculated and expressed in Spearman’s r coefficients.

A multiple linear regression analysis was performed to evaluate the association between various predictors and tHcy concentration. We investigated the possible interaction between the plasma folate, vitamin B-12, and creatinine concentrations and age by including an interaction term between age and the variables in a multiple linear regression model. In this model, tHcy was the dependent variable, and age, plasma folate, vitamin B-12, and creatinine concentrations were the independent variables. The β coefficients express the changes in log-transformed plasma tHcy (µmol/L) that are associated with a 1-unit change in both log-transformed plasma folate (nmol/L) and plasma vitamin B-12 (µmol/L). Because of this logarithmic transformation of both the x variable and the y variable, the interpretation of these coefficients is as follows: a 1% change in the x variable corresponds to a β% change in the y variable. To test whether the relation between plasma folate and tHcy was significantly modified by age—ie, that the differences between the β coefficients across the age groups were significant—we added the folate × age group interaction term to the regression model.

Seven children in the youngest group (aged 0–1 y) were tested for plasma folate, vitamin B-12, and creatinine concentrations. No firm conclusions could be drawn from this small subset of samples, and consequently those 7 subjects were not included in this analysis. The association of tHcy with plasma folate and vitamin B-12 was also shown graphically for evaluation of the slope direction as a function of concentration range. To test whether the slope directions were significantly different below and above the plasma folate and vitamin B-12 concentration cutoffs, we added to the linear regression model a dichotomous variable for high or low folate and high or low vitamin B-12 as an interaction term.

Age × genotype interaction was investigated in a linear regression model by adding an interaction term age × genotype describing the relation between MTHFR 677C→T polymorphism and tHcy concentration. The geometric mean concentration of tHcy was calculated by MTHFR genotypes (CC, CT, and TT).

RESULTS

Characteristics of the subjects

A total of 234 white children participated in this study—115 males and 119 females. The mean age of the study group was 8.4 y (range: 0–19 y). Geometric means (and 95% CIs) for tHcy,
plasma folate, vitamin B-12, and creatinine concentrations in the age groups are shown in Table 1. The geometric mean tHcy concentration for the total population (n = 234) was 6.2 μmol/L (95% CI: 5.9, 6.6). We observed a wide range of tHcy concentrations in the newborns (aged 0 y).

For both boys and girls, the geometric mean tHcy concentrations increased significantly as a function of age. For the whole cohort, no significant interaction between age and sex was present (P = 0.7). The concentrations in boys reached adult values at age 15 y.

Both plasma folate and vitamin B-12 concentrations decreased significantly with age. The plasma creatinine concentration increased with age (Table 1). No difference was seen between the boys and the girls in these variables. Very high concentrations of plasma folate were measured in 7 of the youngest children (aged 0–1 y), whose mean value of 79 nmol/L (95% CI: 60, 104) was 3 to 5 times that in the older children. The plasma vitamin B-12 concentration measured in the youngest children was 439 pmol/L (95% CI: 326, 591 pmol/L), which was not significantly lower than that in the 2–5-y-old children (497 pmol/L; 95% CI: 441, 560 pmol/L) but was twice that in the oldest children (aged 15–19 y).

Change in tHcy concentrations in relation to plasma folate, vitamin B-12, and creatinine

Predictors of tHcy concentration were estimated by multiple linear regression analysis (Table 2). In the regression model, the plasma folate × age interaction for the continuous relation between tHcy and plasma folate concentration was significant (P = 0.003); the plasma vitamin B-12 × age interaction for the relation between tHcy and plasma vitamin B-12 and the plasma creatinine × age interaction for the relation between tHcy and creatinine were not significant (P = 0.2 and 0.6, respectively). Because of the significant interaction, the β coefficients were stratified to age for plasma folate. The relation between tHcy and plasma folate, after adjustment for plasma vitamin B-12 and creatinine, was negative for all age groups (Table 2). The slopes of the tHcy and plasma folate relation and those adjusted for vitamin B-12 were significantly different across the age groups. After adjustment for vitamin B-12 and creatinine, the differences in slope between the 2–5-y-old group and the 11–14- and 15–19-y-old groups and between the 6–10-y-old group and the 15–19-y-old group remained significant. The β coefficient of the relation between tHcy and plasma vitamin B-12, after adjustment for age, plasma folate, and creatinine, was −0.16 (95% CI: −0.26, −0.05) for the whole group.

To evaluate the slope direction as a function of concentration range, we plotted the tHcy concentrations against plasma folate (Figure 1) and plasma vitamin B-12 (Figure 2) for the whole group. The plots show that elevated tHcy concentrations seemed to be most frequent when the plasma folate concentration was <20 nmol/L and when the plasma vitamin B-12 concentration was <200 pmol/L. At higher concentrations of plasma folate and vitamin B-12, the dose-response relation between vitamins and tHcy appeared to plateau. We tested the significance of this observation by adding these cutoffs to the regression model. The additional plasma folate × low or high (≤20 nmol/L) folate interaction was significant (P = 0.03), but the additional plasma

### Table 1

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Plasma folate (nmol/L)</th>
<th>Vitamin B-12 (pmol/L)</th>
<th>Creatinine (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–1 y</td>
<td>46.2 (45.6, 46.8)</td>
<td>497 (441, 560)</td>
<td>62 (59, 66)</td>
</tr>
<tr>
<td>2–5 y</td>
<td>5.1 (4.6, 5.6)</td>
<td>439 (326, 591)</td>
<td>41 (33, 49)</td>
</tr>
<tr>
<td>6–10 y</td>
<td>5.2 (4.7, 5.7)</td>
<td>497 (441, 560)</td>
<td>41 (33, 49)</td>
</tr>
<tr>
<td>11–14 y</td>
<td>6.3 (5.8, 6.8)</td>
<td>389 (345, 438)</td>
<td>41 (33, 49)</td>
</tr>
<tr>
<td>15–19 y</td>
<td>7.4 (6.9, 8.0)</td>
<td>318 (284, 355)</td>
<td>41 (33, 49)</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Age Group</th>
<th>tHcy versus plasma folate</th>
<th>Adjusted for plasma vitamin B-12</th>
<th>Adjusted for plasma vitamin B-12 + creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–5 y (n = 43)</td>
<td>−0.42 (−0.52, −0.30)</td>
<td>−0.39 (−0.50, −0.29)</td>
<td>−0.32 (−0.42, −0.21)</td>
</tr>
<tr>
<td>6–10 y (n = 43)</td>
<td>−0.36 (−0.48, −0.24)</td>
<td>−0.34 (−0.46, −0.22)</td>
<td>−0.29 (−0.40, −0.18)</td>
</tr>
<tr>
<td>11–14 y (n = 50)</td>
<td>−0.31 (−0.43, −0.18)</td>
<td>−0.30 (−0.42, −0.18)</td>
<td>−0.27 (−0.38, −0.15)</td>
</tr>
<tr>
<td>15–19 y (n = 46)</td>
<td>−0.25 (−0.37, −0.12)</td>
<td>−0.25 (−0.37, −0.13)</td>
<td>−0.24 (−0.36, −0.12)</td>
</tr>
</tbody>
</table>

1 All values are geometric x; 95% CI in parentheses. All variables were measured in plasma.

2 Spearman’s correlation test was used to establish the relation between tHcy, plasma folate, vitamin B-12, and creatinine concentrations and age. P for trend was significant (P < 0.0001) for all correlation coefficients.

3 n = 48, 45, 44, 51, and 46 for the age groups from left to right. Sex × age interaction was not significant (P = 0.7).

4 Calculated in a linear regression model.

5 N = 7, 43, 43, 50, and 46 for the age groups from left to right.
vitamin B-12 × low or high (≤ or ≥ 200 pmol/L) vitamin B-12 interaction was not significant (P = 0.4).

A significant positive linear association was observed between tHcy and plasma creatinine (r = 0.57, P < 0.0001). In the multiple linear regression model, after adjustment for age, plasma folate, and vitamin B-12 concentrations, the effect of plasma creatinine on the tHcy concentration was small but significant (β = 0.005, 95% CI: 0.001, 0.01).

The influence of MTHFR 677C→T polymorphism on tHcy concentration

Of the 220 subjects, 18 (8.2%) were homozygous (TT), 104 (47.3%) were heterozygous (CT), and 98 (44.5%) were wild-type (CC) for the 677C→T polymorphism in the MTHFR gene. The genotype × age interaction in the regression model was not significant (P = 0.13). For all ages, the geometric mean tHcy concentrations were 6.2 (95% CI: 5.8, 6.7), 6.3 (5.8, 6.8), and 6.8 μmol/L (5.2, 8.5 μmol/L) for the CC, CT, and TT genotypes, respectively. The P values for the effect of MTHFR 677CT and TT genotypes on tHcy concentrations were 0.7 and 0.3, respectively.

To investigate the effect of the MTHFR 677C→T polymorphism on tHcy concentrations in relation to plasma folate concentrations, we included plasma folate × genotype as independent variables in the linear regression model. The genotype × plasma folate interaction, adjusted for age, was significant (P = 0.03).
Meidication, puberty, and smoking

None of the children in this study used medication that could interfere with folate metabolism. We investigated the possible effect of puberty on tHcy concentrations in adolescents. As the criterion for exposure to endogenous sex hormones in children aged 10–19 y, we used the occurrence of menarche or the growth of beard hair. In the relatively small subgroups, no significant difference in the tHcy concentration was found between children with or without these features (data not shown).

Seven children in the oldest group (aged 15–19 y) smoked cigarettes. The geometric mean tHcy concentration in the smokers group was 9.0 μmol/L (95% CI: 7.3, 11.1 μmol/L). This did not differ significantly from the 8.6 μmol/L (95% CI: 7.9, 9.4 μmol/L) seen in nonsmokers.

DISCUSSION

This study was primarily designed to explore tHcy and its predictors (ie, age; sex; plasma folate, vitamin B-12, and creatinine concentrations; and MTHFR genotype) in presumably normal white children aged 0–19 y. The mean tHcy concentrations in these Dutch children ranged from 4.6 to 8.7 μmol/L, which is comparable to those observed in other European children (19, 20, 23, 26, 27, 29, 30, 32, 34, 35). Studies performed in black and white children in the United States or Canada found tHcy concentrations ≈1.5 μmol/L lower than our values (21, 22, 28, 31, 33). These differences in tHcy concentrations may indicate a real difference between populations due to environmental, including nutritional and genetic, factors. Must et al (31) observed differences in tHcy concentrations among 3 racial or ethnic groups of children.

The strong association that we observed between tHcy concentration and age was also found in all earlier studies except that of Reddy et al (34). Several studies established age subgroups of beard hair. In the relatively small subgroups, no significant difference in the tHcy concentration found to be significant at ⩾10 y of age (31). Both the higher tHcy concentration in boys than in girls and the age effect could be explained by increases in muscle mass according to age and sex. This contention is supported by studies showing a positive relation between tHcy and creatinine in healthy children without renal dysfunction (19, 29). We observed a small but significant effect of plasma creatinine on tHcy concentration. Considering the negative correlation between estrogen concentrations and tHcy concentrations, the postpubertal differences in tHcy concentration may also be explained by exposure to estrogen in pubescent girls (41).

Our observation that both plasma folate and vitamin B-12 concentrations decreased markedly with age has also been described in 3 other studies (27, 35, 42). The high plasma vitamin concentrations may be reflected in the lower tHcy concentrations at younger age. In the current study, plasma folate is inversely correlated with tHcy concentrations at all ages, which is consistent with findings in previous studies in children (13–15, 19, 20, 22–27, 29, 30, 35, 36). Elevation of tHcy was particularly seen when the plasma folate concentration was <20 nmol/L. This meant that 29, 60, 71, and 63% of the 2–5-, 6–10-, 11–14-, and 15–19-y-olds, respectively, were at risk of elevated tHcy. Osganian et al (22) showed in 13–14-y-old US children that tHcy increased at slightly higher concentrations of plasma folate, starting at ≈30 nmol/L.

Other studies observed an significant inverse relation between tHcy and plasma vitamin B-12 concentrations; age was not always evaluated (20, 22, 23, 25, 27, 29, 30, 35, 36). In our study, elevation of tHcy seemed to occur when plasma vitamin B-12 concentration was <200 pmol/L. Persons with a vitamin B-12 concentration significantly <200 pmol/L had higher tHcy concentrations, but they did not differ significantly from those in persons with vitamin B-12 200 pmol/L. This meant that 4, 4, 2, and 28% of the 2–5-, 6–10-, 11–14-, and 15–19-y-olds, respectively, were at risk of elevated tHcy. In the third National Health and Nutrition Examination Survey, which included adolescents (>12 y old) and adults, approximately two-thirds of the cases of high tHcy concentration were associated with low plasma folate concentrations and with vitamin B-12 concentrations < 250 pmol/L (43). Folate and vitamin B-12 supplementation, given to lower tHcy concentrations, may be less effective if plasma folate and vitamin B-12 concentrations are >200 pmol/L and >200 pmol/L, respectively. This is relevant because hyperhomocysteinemia is also a known risk factor for ischemic stroke in children (5–8). Intervention studies are needed to illuminate this issue.

We also observed a greater variation in tHcy concentrations in newborns during the first months. Newborns, in particular those who were premature, should be considered a separate group for whom separate reference ranges should be established (15–17). The reports evaluating tHcy in newborns showed that, in a significant proportion of newborns, elevated tHcy could be attributed to an impaired vitamin B-12 status, and that vitamin B-12 deficiency and higher tHcy and methylmalonic acid concentrations were frequently present in breastfed babies (13, 14, 17). In our study, the number of blood samples for vitamin concentration measurement (n = 7) was too low to evaluate this relation.

The proportion of children identified with the MTHFR 677TT genotype (8.2%) was lower than that reported in French Canadian children (17%) by Delvin et al (36). Balasa et al (33) reported the MTHFR 677TT genotype to be present in 11% of whites and 3% of African Americans. In our study, the MTHFR
677C→T polymorphism did not significantly influence the tHcy concentration overall. Higher tHcy concentrations were confined to persons with the TT genotype and lower plasma folate status, which was comparable to observations in adults (11, 40, 44). High plasma folate concentrations, particularly in younger children, may prevent elevated tHcy concentrations in those with the MTHFR 677TT genotype. Balasa et al (33) found that the MTHFR 677TT genotype accounted for 2.9% of the variance in tHcy in children, but data about folate status were absent. In the older children (age > 10 y) in the study by Delvin et al (36), the MTHFR 677TT genotype resulted in lower plasma folate concentrations with a trend toward higher tHcy concentrations.

In summary, we provided data on age-specific tHcy concentrations and their predictors in white children aged 0–19 y. Considering the growing interest in tHcy as a risk factor for cardiovascular disease in children (5–9) and in relation to other diseases (45), it is important to provide age-specific data and to explore the predictors of tHcy concentration. In the current study, tHcy concentrations were strongly related to age and were lower than those seen in adults in other studies. Plasma folate and vitamin B-12 concentrations were predictors of the plasma tHcy concentrations in children. The influence of plasma creatinine on tHcy was small, but present. The MTHFR 677C→T polymorphism did not significantly influence tHcy, except in children with low plasma folate status.

We thank all the children for their participation. IMvE was responsible for data collection, data analysis, and writing of the manuscript. Mdk is recipient of a VENI grant from the Netherlands Foundation of Scientific Research and was involved in the study design, data analysis and manuscript review. CMGT was responsible for vitamin analysis and manuscript preparation. LA performed the genotyping and data analysis. Do-vE was involved in data collection and tHcy determination. HJB is an Established Investigator of the Netherlands Heart Foundation, was the principal investigator, and was involved in all aspects of the study. None of the authors had any financial or personal interest in the Netherlands Heart Foundation or any other conflict of interest.

REFERENCES


Diabetes mellitus worsens antioxidant status in patients with chronic pancreatitis1–3

Didier Quilliot, Evelyne Walters, Jean-Paul Bonte, Jean-Charles Fruchart, Patrick Duriez, and Olivier Ziegler

ABSTRACT

Background: Patients with chronic pancreatitis (CP) are at high risk of antioxidant deficiencies. Furthermore, this disease can lead to diabetes mellitus (DM) that could exacerbate the severity of oxidative stress. Oxidative stress and the resulting LDL oxidation are a major cause of atherosclerosis.

Objective: The objective of the study was to ascertain whether diabetes significantly modifies oxidative status in patients with CP.

Design: CP patients with or without DM were compared with type 1 DM patients and healthy control subjects.

Results: Two-way factorial analyses showed that a decrease in the plasma concentrations of vitamin A, vitamin E, and carotenoids accompanied both CP and DM, and CP was also associated with lower plasma concentrations of selenium and zinc, lower catalase activity, and higher plasma concentrations of copper. The lag phase of LDL oxidation was lower in CP patients with or without DM than in the control subjects, whereas there was no significant difference between type 1 DM patients and control subjects. Multivariate analysis showed that LDL vitamin E ($R^2 = 0.24$, $P < 0.0001$) and fasting plasma glucose ($R^2 = 0.32$, $P < 0.0001$) concentrations were the main determinants of the lag phase of LDL oxidation.

Conclusions: Antioxidant status is altered in CP patients, particularly in those who also have DM. In these patients, a vitamin E deficiency and an elevated plasma glucose concentration were associated with significantly higher LDL oxidizability. Am J Clin Nutr 2005;81:1117–25.

KEY WORDS Chronic pancreatitis, diabetes mellitus, antioxidant, oxidized LDL, immune complexes, malondialdehyde

INTRODUCTION

Patients with exocrine pancreatic insufficiency are at a greater risk than are patients with exocrine pancreatic sufficiency of developing vitamin or trace-element deficiencies as a result of malabsorption (1–6). This finding is mostly in relation to the fat-soluble vitamins, although the role of water-soluble vitamins—particularly vitamin C—in this disease has been studied only sparingly (7). Some vitamins, such as vitamins E, C, and A and the carotenoids, have antioxidant properties, as do the trace elements selenium and zinc. The prevalence of lower plasma concentrations of these vitamins and trace elements in patients with chronic pancreatitis (CP) has been reported (8, 9). Moreover, lower concentrations of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, were implicated in cases of recurrent and acute pancreatitis (10, 11), which means that antioxidant deficiencies could have several serious consequences in that disease (12). Because of a heightened oxidative modification of LDL in CP (13), accelerated atherosclerosis could be one of several complications associated with this condition (14). It is surprising that, despite a low LDL-cholesterol concentration in patients with diabetes secondary to CP, those patients have the same prevalence and distribution of atherosclerosis as do patients with type 1 diabetes mellitus (DM) (15–17).

Early in vitro and in vivo trials of antioxidant supplements showed that antioxidants increase the resistance of LDL to oxidation (18). However, Estebauer et al. (19) showed in vitamin E–nondeficient subjects that the resistance of LDL to oxidation, as ascertained by the duration of the lag phase in copper ion–induced oxidation, did not correlate with the LDL α-tocopherol content. In contrast, the nature of the relation between LDL oxidation resistance and LDL α-tocopherol content in patients with severe antioxidant deficiencies is not known. LDL fatty acid abnormalities, which we described previously in relation to type 2 diabetes mellitus (20), could also be responsible for modifying LDL oxidizability. Because of the antigenic properties of oxidized LDL, antibodies to oxidized LDL could serve as a useful index of in vivo LDL oxidation. Furthermore, LDL immune complexes (ICs) could play a role in atherogenesis (21). DM secondary to CP could exacerbate the severity of antioxidant deficiencies, given that DM accelerates Cu²⁺-induced ex vivo LDL oxidation (although these data are controversial; 22), whereas, in type 1 DM, the excess risk of atherosclerosis persists after control for lipidic factors (eg, LDL and HDL cholesterol) (23, 25). DM is also associated with deficiencies in vitamins and trace elements, particularly those of vitamin C (25–28), vitamin E (29, 30), zinc (8, 31, 32), and SOD (33).

1 From the Service de Diabétologie, Maladies Métaboliques et Nutrition, Hôpital Jeanne d’Arc, CHU de Nancy, France (DQ and OZ); the Centre d’Investigation Clinique, CHU de Nancy, France (DQ); the Centre Universitaire de Mesure et d’Analyse, Faculté de Pharmacie, Université de Lille II, Lille, France (J-PB); and INSERM U325, Département d’Athérosclérose, Institut Pasteur de Lille et Faculté de Pharmacie, Université de Lille II, Lille, France (EW, J-CF, and PD).

2 Supported by Fournier Laboratory (Dijon, France).

3 Address reprint requests to D Quilliot, Service de Diabétologie, Maladies Métaboliques et Nutrition, Hôpital Jeanne d’Arc, CHU Nancy, 54200 Toul cedex, France, E-mail: d.quilliot@chu-nancy.fr.

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The main objectives of this study were to ascertain whether diabetes significantly modifies the oxidative status of patients with CP and to ascertain the extent of Cu$^{2+}$-induced ex vivo LDL oxidation and the concentrations of antibodies to oxidized LDL and LDL-ICs under low LDL-cholesterol conditions. We thus examined these variables in patients with DM secondary to CP and made a comparative analysis with findings in type 1 DM patients, patients with CP but without diabetes, and healthy control subjects.

SUBJECTS AND METHODS

Subjects

Ninety-two male subjects were classified into 4 groups: CP patients without diabetes (CP nondiabetic group; n = 12), patients with diabetes secondary to CP or insulin-treated diabetes (CP diabetic group; n = 35), patients with type 1 DM (control diabetic group; n = 25), and healthy control subjects (control nondiabetic group; n = 20). The patients with type 1 DM were individually matched with the CP diabetic patients for age and diabetes duration ($\pm 2$ y). The control subjects were matched for social category and age with the CP nondiabetic patients (Table 1).

CP was diagnosed on the basis of clinical history and the presence of morphologic pancreatic abnormalities, in particular that of pancreatic calcifications detected by X-ray and confirmed by abdominal ultrasonography, computerized tomography, or echoendoscopy. All CP diabetic patients and 10 of the 12 CP nondiabetic patients had calcific pancreatitis. One patient was diagnosed by using an ultrasonography scanner and another by using echoendoscopy.

All patients in the CP diabetic group had ceased or greatly reduced alcohol intake by the time of the study. On average, they had consumed $<30$ g alcohol/d for the 5 y immediately before the study. Their previous alcohol consumption averaged 111 $\pm 66$ g/d (range: 50–450 g/d) for a period of 25.5 $\pm 11.4$ y. Three of the CP diabetic patients had undergone a cholecdochojunostomy, and 4 had undergone cystic derivations. Patients who had undergone pancreatectomy or gastrectomy were excluded.

All patients in the CP diabetic group had diabetes according to the criteria of the American Diabetes Association (34, 35) and had been taking insulin for $\geq 3$ mo. No patient had an episode of acute pancreatitis in the 3 mo before the study. All the patients in the CP nondiabetic group had a normal fasting plasma glucose concentration—ie, $<110$ mg/L—on the day they entered the study.

Eleven of the 12 CP nondiabetic patients and 20 of the 35 CP diabetic patients had had or were currently having recurrent attacks of pain (painful pancreatitis). Fifteen of the 35 CP diabetic patients had a family history of diabetes.

Patients being treated with pancreatic enzymes underwent a 5-d washout period. CP patients on a home-based diet provided a 72-h stool sample after the wash-out period. Blood samples were collected after the overnight fast, before the insulin injection, and on day 6 of the washout period. None of the patients or control subjects received additional vitamin or trace element supplementation or had been assigned to a special diet.

In addition to a physical examination and an antioxidant investigation, each subject with DM underwent the following investigation: evaluation of the major vascular risk factors [ie, blood pressure, smoking status, and body mass index (BMI; in kg/m$^2$)], electrocardiogram, and continuous-wave Doppler velocimetry.

Diabetic patients underwent an ophthalmologic examination and retinal fluorescein angiography.

Written informed consent was obtained from all subjects. The study was approved by the Nancy University ethics committee for the Protection of Human Subjects.

Analytic methods

Plasma concentrations of glucose were measured by using the glucose oxidase method in an automated glucose analyzer. HPLC (Diamat; BioRad Laboratories, Hercules, CA) was used to determine concentrations of glycated hemoglobin (HbA$\text{$_1$C}$), which were expressed as a percentage of total hemoglobin (normal range: 4.5–6%).

The fecal fat content in patients in the CP patient groups was analyzed according to Van de Kamer et al (36). HDL-cholesterol concentrations were obtained after the precipitation of apolipoprotein (apo) B-containing lipoproteins with sodium phosphotungstate and magnesium chloride (Boehringer Mannheim, Mannheim, Germany). Total-cholesterol and triacylglycerol concentrations were ascertained by using commercial kits (Boehringer Mannheim) adapted to a Hitachi 911 analyzer (Roche Diagnostics, Indianapolis, IN). LDL-cholesterol concentrations were calculated according to the method of Friedewald et al (37).

Preparation of LDL

Plasma LDL was prepared by ultracentrifugation with a Beckman TL100 centrifuge and a TLA-100.4 rotor (Beckman Instruments, Inc, Palo Alto, CA) within a density cutoff range of 1.019 to 1.063 g/mL. EDTA (1 mg/mL) was added to plasma before ultracentrifugation. First the LDL was dialyzed against sodium chloride (0.15 mol/L) in EDTA (1 mg/mL) with the pH adjusted to 7.4; next it was gassed with N$_2$, and then it was sterilized by filtration (0.45 $\mu$m) and stored at 4 °C. Apo B concentrations were analyzed with an immunonephelometric assay on a laser reader and by using commercial polyclonal antibodies (both: Behring, Marburg, Germany).

Fatty acid composition of LDL and lipoprotein chemical composition

The fatty acid composition of LDL was ascertained after lipid extraction (38). Lipids were methanolized with methanolic H$_2$SO$_4$ for 2 h at 70 °C. Fatty acid methyl esters were extracted with the use of heptane and analyzed by gas chromatography on a Varian 3400 chromatograph (Sunnyvale, CA) equipped with a flame ionization detector and a capillary wall-coated open tubular fused Silica 88 column containing cyanopropylsilsiloxane (Chrompack, Middelburg, Netherlands). Cholesterol, phospholipids, and triacylglycerol were analyzed in VLDL, LDL, and HDL after separation by sequential ultracentrifugation.

Analysis of LDL oxidation, malondialdehyde-modified LDL antibodies, and LDL immune complexes

LDL oxidation was studied in 65 subjects. LDL was dialyzed against phosphate-buffered saline that had been gassed with N$_2$, containing no EDTA. Oxidation was induced by adding CuCl$_2$ (final concentration: 1.66 $\mu$mol/L) to 100 $\mu$g LDL/mL (apo B). This mixture was incubated at 30 °C, and conjugated diene formation was followed by the measurement of absorption at 234 nm every 10 min for 8 h with a thermostat-controlled Kontron
UVikon 930 spectrophotometer equipped with a 10-position sample changer (Tegimenta AG, Rotkreuz, Switzerland). Analyses were performed in triplicate and expressed in nmol·min⁻¹·mg⁻¹ of apo B. Malondialdehyde (MDA)-modified LDL antibodies and LDL-ICs were analyzed according to the method of Gunzler et al (44). Catalase activity was determined spectrophotometrically at 240 nm by using the method of Beers and Sizer (45) and was expressed in Bergmeyer units (1 U = 1 nmol of H₂O₂/min at 25 °C; normal range: 60–100 U/ml) (Beckman, Brea, CA).

**Assessment of vitamins and trace elements**

Serum vitamin A (normal range: 240–900 µg/L) and carotenoid concentrations were measured simultaneously by reversed-phase HPLC as described by Steghens et al (40); the normal range for β-carotene, α-tocopherol, and lycopene was 100–480, 27–200, and 50–380 µg/L, respectively. Measurement of vitamin E (α-tocopherol) in plasma and in LDL was performed by HPLC according to the method of Teissier et al (41) and by using a 600 E controller equipped with a refrigerated 717-plus autosampler and a Novapack C18 column and precolumn (all: Waters Associates, Milford, MA). Plasma concentrations of ascorbic acid (normal range: 4–14 mg/L) were measured by using the 2,4-dinitrophenylhydrazine method with HPLC and electrochemical detection. Atomic absorption spectrophotometry was used to measure the concentrations of zinc (normal range: 0.7–1.1 mg/L), copper (normal range: 0.8–1.6 mg/L), and selenium (normal range: 60–83 µg/L) by the method of Clavel et al (42).

**Antioxidant enzymes**

The concentration of the Cu-Zn-SOD enzyme in plasma (normal range: 2300–3800 UI/g hemoglobin) was ascertained by using an AbA-200 direct chromatic analyzer (Abbott Laboratories, Irving, TX) and the technique of L’Abbe and Fisher (43). Plasma and erythrocyte concentrations of glutathione peroxidase were assayed by using the Ellman reaction as modified by Gunzler et al (44). Catalase activity was determined spectrophotometrically at 240 nm by using the method of Beers and Sizer (45) and was expressed in Bergmeyer units (1 U = decomposition of 1 g H₂O₂/min at 25 °C; normal range: 60–100 U/ml) (Beckman, Brea, CA).

**Statistical analysis**

Statistical analyses were performed with BMDP statistical software (version 7.0; BMDP, Los Angeles, CA). Results are expressed as mean (±SD), with data tested for normality by skewness and kurtosis tests. The 4 groups of patients were compared by using factorial analyses [2 × 2 analysis of variance (ANOVA)] and a Sheffe’s F test if the interaction was significant. Chi-square tests were used to compare the frequencies of vitamin and trace element deficiencies among the 4 groups.

Pearson’s correlation was used for testing 2-variable relations. Multivariate linear regressions were performed to ascertain the effects of certain factors on antioxidant concentrations. In the 2 subgroups of subjects with CP, multivariate linear regressions were performed to adjust on for the effect of diabetes. In the CP diabetic subgroup, multivariate linear regressions were performed to adjust for the effect of CP. Multiple regression analysis was also used to assess the effects of several independent variables on lag phase. Age, tobacco consumption, and alcohol intake had no independent effect in any of the analyses. P values of < 0.05 were considered significant.

**RESULTS**

Subjects’ baseline characteristics

The anthropometric characteristics of the 4 groups of subjects showed that the CP groups (diabetic and nondiabetic) had significantly lower body weight and BMI values than did the control diabetic and control nondiabetic groups (Table 1). CP duration and the amount of steatorrhea were significantly less in CP nondiabetic than in CP diabetic patients. Pancreatitis duration was 8 ± 5.2 y in the CP nondiabetic group and 16.3 ± 7.7 y in the CP diabetic group (P < 0.001), and steatorrhea was 5.5 ± 5.2 g/d in the CP nondiabetic group and 12.0 ± 8.4 g/d in the CP diabetic group (P = 0.028).

The prevalence of macroangiopathy was 12% (3/25) in the control diabetic group and 28.5% (10/35) in the CP diabetic group (NS; chi-square test). The prevalence of retinopathy was
38% (8/25) in the control diabetic group and 40% (14/35) in the CP diabetic group (NS; chi-square test).

Among the CP patients, 11 of the 12 CP nondiabetic and 20 of the 35 CP diabetic patients had had bouts of pain. There was no significant difference between patients with or without pain in terms of age, diabetes and pancreatitis duration, steatorrhea, Hb, hemoglobin, HbA1c, and BMI. The number of bouts of pancreatitis was significantly greater (P < 0.001) and significantly lower in the CP diabetic group than in the control diabetic group (P < 0.05). Vitamin E concentrations were significantly lower in the control diabetic group than in the control nondiabetic group (P < 0.01). The prevalence of a low plasma vitamin E concentration—ie, below the lower limit of the normal range, <7 mg/L—was 71% in the CP diabetic group and 16% in the CP nondiabetic group. No control diabetic patient presented with a vitamin E concentration below this value (P < 0.0001; chi-square test). Compared with that in the control nondiabetic group, the LDL vitamin E concentration was significantly lower in both CP groups than in the control nondiabetic group; in addition, it tended to be lower in the control diabetic group than in the control nondiabetic group, but this latter difference was not significant.

Plasma concentrations of vitamin E and carotenoid (ie, α-carotene, β-carotene, lycopene) were significantly lower in both CP groups than in the control nondiabetic group (P < 0.001) and significantly lower in the CP diabetic group than in the control diabetic group (P < 0.05). Vitamin A concentrations were significantly lower in the control diabetic group than in the control nondiabetic group (P < 0.01). The prevalence of a low plasma vitamin E concentration—ie, below the lower limit of the normal range, <7 mg/L—was 71% in the CP diabetic group and 16% in the CP nondiabetic group. No control diabetic patient presented with a vitamin E concentration below this value (P < 0.0001; chi-square test). Compared with that in the control nondiabetic group, the LDL vitamin E concentration was significantly lower in both CP groups than in the control nondiabetic group; in addition, it tended to be lower in the control diabetic group than in the control nondiabetic group, but this latter difference was not significant.

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Plasma concentrations of vitamin A and carotenoid (ie, α-carotene, β-carotene, lycopene) were significantly lower in both CP groups than in the control nondiabetic group (P < 0.001) and significantly lower in the CP diabetic group than in the control diabetic group (P < 0.05). Vitamin E concentrations were significantly lower in the control diabetic group than in the control nondiabetic group (P < 0.01). The prevalence of a low plasma vitamin E concentration—ie, below the lower limit of the normal range, <7 mg/L—was 71% in the CP diabetic group and 16% in the CP nondiabetic group. No control diabetic patient presented with a vitamin E concentration below this value (P < 0.0001; chi-square test). Compared with that in the control nondiabetic group, the LDL vitamin E concentration was significantly lower in both CP groups than in the control nondiabetic group; in addition, it tended to be lower in the control diabetic group than in the control nondiabetic group, but this latter difference was not significant.

### TABLE 2
Plasma vitamin and carotenoid concentrations

<table>
<thead>
<tr>
<th>Subject group</th>
<th>Control nondiabetic (n = 20)</th>
<th>Control diabetic (type 1 DM) (n = 25)</th>
<th>CP nondiabetic (n = 12)</th>
<th>CP diabetic (n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E (mg/L)</td>
<td>17.4 ± 3.7&lt;sup&gt;4&lt;/sup&gt;</td>
<td>12.9 ± 2.9&lt;sup&gt;4&lt;/sup&gt;</td>
<td>10.7 ± 2.9&lt;sup&gt;4&lt;/sup&gt;</td>
<td>5.8 ± 2.3&lt;sup&gt;4,6&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDL vitamin E (mg/g)</td>
<td>6.4 ± 1.3</td>
<td>3.7 ± 1.3</td>
<td>3.2 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>α-Carotene (mg/L)</td>
<td>0.398 ± 0.275</td>
<td>0.307 ± 0.192</td>
<td>0.108 ± 0.091</td>
<td>0.067 ± 0.069</td>
</tr>
<tr>
<td>Lycopene (mg/L)</td>
<td>232.3 ± 114.1</td>
<td>126.6 ± 101.3</td>
<td>120.0 ± 133.5</td>
<td>68.0 ± 59.2</td>
</tr>
<tr>
<td>β-Carotene (mg/L)</td>
<td>165.8 ± 128.1</td>
<td>108.8 ± 82.6</td>
<td>70.8 ± 50.2</td>
<td>47.7 ± 40.4</td>
</tr>
<tr>
<td>Vitamin A (µg/L)</td>
<td>1028 ± 284</td>
<td>801 ± 316</td>
<td>682 ± 296</td>
<td>537 ± 215</td>
</tr>
<tr>
<td>Vitamin C (mg/L)</td>
<td>9.30 ± 4.46</td>
<td>8.80 ± 4.37</td>
<td>8.57 ± 6.71</td>
<td>7.33 ± 4.67</td>
</tr>
</tbody>
</table>

<sup>1</sup> SOD, superoxide dismutase; CP nondiabetic, chronic pancreatitis without diabetes; CP diabetic, CP with insulin-treated diabetes mellitus; DM, diabetes mellitus.
<sup>2</sup> ANOVA 2 x 2 with Scheffe’s test if the interaction was significant.
<sup>3</sup> Mean ± SD (all such values).
<sup>4</sup> Significantly different from the control nondiabetic group, P < 0.001.
<sup>5</sup> Significantly different from the control diabetic group, P < 0.05.
<sup>6</sup> Significantly different from the control nondiabetic group, P < 0.001.

### TABLE 3
Plasma trace element concentrations and enzyme activities

<table>
<thead>
<tr>
<th>Subject group</th>
<th>Control nondiabetic (n = 20)</th>
<th>Control diabetic (type 1 DM) (n = 25)</th>
<th>CP nondiabetic (n = 12)</th>
<th>CP diabetic (n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc (mg/L)</td>
<td>0.97 ± 0.01&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0.94 ± 0.13</td>
<td>0.88 ± 0.18</td>
<td>0.85 ± 0.15</td>
</tr>
<tr>
<td>Copper (mg/L)</td>
<td>0.95 ± 0.20</td>
<td>1.12 ± 0.15&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.16 ± 0.12&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.13 ± 0.17&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Zn:Cu</td>
<td>1.04 ± 0.30</td>
<td>0.85 ± 0.25&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.75 ± 0.13&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.78 ± 0.20&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Selenium (µg/L)</td>
<td>84.6 ± 14.8</td>
<td>82.3 ± 14.2</td>
<td>62.2 ± 11.5</td>
<td>64.8 ± 14.2</td>
</tr>
<tr>
<td>SOD (U/L)</td>
<td>2841 ± 332</td>
<td>2628 ± 344</td>
<td>2607 ± 382</td>
<td>2811 ± 589</td>
</tr>
<tr>
<td>Erythrocyte glutathione peroxidase (U/g Hb)</td>
<td>6.39 ± 1.25</td>
<td>5.86 ± 1.08</td>
<td>6.04 ± 1.25</td>
<td>5.97 ± 2.02</td>
</tr>
<tr>
<td>Plasma glutathione peroxidase (U/mL)</td>
<td>80.1 ± 24.2</td>
<td>82.8 ± 26.9</td>
<td>74.9 ± 17.8</td>
<td>81.5 ± 27.2</td>
</tr>
<tr>
<td>Catalase (U/mL)</td>
<td>81.7 ± 8.0</td>
<td>79.1 ± 9.3</td>
<td>73.4 ± 7.8</td>
<td>76.8 ± 9.9</td>
</tr>
</tbody>
</table>

<sup>1</sup> CP nondiabetic, chronic pancreatitis without diabetes; CP diabetic, CP with insulin-treated diabetes; DM, diabetes mellitus; SOD, superoxide dismutase; Hb, hemoglobin.
<sup>2</sup> ANOVA 2 x 2 with Scheffe’s test if the interaction was significant.
<sup>3</sup> Mean ± SD (all such values).
<sup>4</sup> Significantly different from control nondiabetic group, P < 0.01.
CP subjects than in control subjects \( (P < 0.01) \). Vitamin A and lycopene concentrations were also significantly lower in diabetic subjects than in nondiabetics \( (P < 0.01) \). The prevalence of a low \( \beta \)-carotene concentration—ie, below the lower limit of the normal range, \( 0.100 \text{ mg/L} \)—was \( 50\% \) in the CP nondiabetic group, \( 74\% \) in the CP diabetic group, and \( 16\% \) in the control diabetic group. No control nondiabetic subjects had such a low concentration \( (P < 0.0001) \); chi-square test).

There was no significant difference in average ascorbate concentrations among the 4 groups. The prevalence of vitamin C deficiency—ie, vitamin C concentration below the lower limit of the normal range, \( 4 \text{ mg/L} \)—was \( 33.3\% \) in CP nondiabetic patients, \( 28.5\% \) in CP diabetic patients, \( 25\% \) in control diabetic patients, and \( 20\% \) in control nondiabetic subjects (NS; chi-square test).

Plasma concentrations of zinc in the CP subjects were significantly \( (P < 0.01) \) lower than those in the control subjects: \( 17\% \) of CP diabetic and \( 16\% \) of CP nondiabetic subjects had concentrations below the lower limit of normal \( (0.7 \text{ mg/L}) \). No subjects in either of the control groups had such low plasma concentrations of zinc \( (P = 0.035) \); chi-square test). All patients had plasma concentrations of copper in the normal range, but average values were significantly \( (P < 0.01) \) higher in both CP groups and the control diabetic group than in the control nondiabetic group.

Plasma concentrations of selenium were significantly lower in CP patients than in control nondiabetic subjects \( (P < 0.0001) \): \( 33.3\% \) of CP nondiabetic subjects, \( 42.8\% \) of CP diabetic subjects, and \( 12\% \) of control diabetic subjects were selenium deficient \( (<60 \text{ mg/L}) \). No control subjects had such a deficiency \( (P = 0.008) \); chi-square test). Catalase activity was significantly \( (P < 0.05) \) lower in CP subjects than in the control groups, whereas SOD, erythrocyte glutathione peroxidase, and plasma glutathione peroxidase activities did not differ significantly between the groups.

### Analysis of the effect of CP on antioxidant status

For the study population as a whole, multivariate analysis was performed to analyze the effects of CP and diabetes after adjustment for the confounding variables age, alcohol intake (g/d), and number of cigarettes smoked/d. These variables had no significant effect on the analysis. CP was associated with significantly lower plasma concentrations of vitamin E \( (\beta = -6.0 \pm 0.7 \text{ mg/L, } P < 0.0001) \), vitamin A \( (\beta = -289 \pm 58 \text{ mg/L, } P < 0.001) \), carotenoids \( (\alpha\text{-carotene: } \beta = -73 \pm 240 \text{ mg/L, } P = 0.004; \text{ lycopene: } \beta = -94 \pm 33.9 \text{ mg/L, } P = 0.007; \beta\text{-carotene: } \beta = -0.25 \pm 0.04 \text{ mg/L, } P < 0.001) \), and trace elements (zinc: \( \beta = -0.09 \pm 0.03 \text{ mg/L, } P = 0.003; \text{ selenium: } \beta = -19.1 \pm 3.0 \text{ mg/L, } P < 0.001) \).

In the subgroup of subjects with CP, the amount of steatorrhea was negatively correlated with vitamin E and \( \beta\text{-carotene concentrations and positively correlated with SOD concentrations (Table 4). These relations were significant for vitamin E and } \beta\text{-carotene even after adjustment for the effect of diabetes.}

### Analysis of diabetes-specific effects

For the study population as a whole, multivariate analysis was performed to analyze the effects of CP and of diabetes after adjustment for the confounding variables age, alcohol intake (g/d), and number of cigarettes smoked/d. These variables had no significant effect on the analysis. DM was associated with significantly lower plasma concentrations of vitamin E \( (\beta = -3.2 \pm 0.8 \text{ mg/L, } P < 0.0001) \), vitamin A \( (\beta = -190 \pm 62 \text{ mg/L, } P = 0.002) \), and lycopene \( (\beta = -111 \pm 33 \text{ mg/L, } P = 0.001) \) and with nonsignificantly lower plasma concentrations of \( \alpha\text{-carotene: } \beta = -45 \pm 24 \text{ mg/L, } P = 0.06 \) and \( \beta\text{-carotene: } \beta = -0.07 \pm 0.04 \text{ mg/L, } P = 0.07 \).

Univariate analyses of variables from the subgroups of diabetic patients (CP and control diabetic groups) showed that \( \beta\text{-carotene and plasma glutathione peroxidase activities were negatively correlated with the fasting plasma concentration of glucose } (P = 0.002 \text{ and } P = 0.001, \text{ respectively}) \) and that plasma glutathione peroxidase activity was negatively correlated with HbA1c \( (P = 0.014) \) (Table 5).

### Antioxidant status of subjects with macroangiopathy or microangiopathy, or both, and effect of pain state

There were no differences in antioxidant status between diabetic subjects with or without vascular complications, except for the plasma glutathione peroxidase activity, which tended to be lower in subjects with macroangiopathy than in those without macroangiopathy \( (86.4 \pm 28.9 \text{ U/L (} n = 13) \text{ and } 69.9 \pm 12.9 \text{ U/L (} n = 37), \text{ respectively; } P = 0.051) \). The presence or absence of pain was not associated with an impaired or deficient antioxidant status in CP patients. The prevalence of such deficiencies was significantly lower in the CP nondiabetic group \( (1.27 \pm 0.8; n = 12) \) than in the CP diabetic groups [painless CP diabetics \( (n = 15): 2.5 \pm 0.8; P < 0.001; \text{ CP diabetics with pain (} n = 20): 2.34 \pm 1.0; P < 0.05] \).

### Table 4

<table>
<thead>
<tr>
<th></th>
<th>Simple linear regression</th>
<th>Adjusted on DM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( r )</td>
<td>( P )</td>
</tr>
<tr>
<td>Plasma vitamin E</td>
<td>-0.64</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma ( \beta\text{-carotene} )</td>
<td>-0.41</td>
<td>0.013</td>
</tr>
<tr>
<td>Plasma SOD</td>
<td>0.41</td>
<td>0.013</td>
</tr>
</tbody>
</table>

\( ^1 \text{DM, diabetes mellitus; SOD, superoxide dismutase.} \)

### Table 5

<table>
<thead>
<tr>
<th></th>
<th>Fasting plasma glucose</th>
<th>HbA1c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( r )</td>
<td>( P )</td>
</tr>
<tr>
<td>( \beta\text{-Carotene} )</td>
<td>-0.32</td>
<td>0.002</td>
</tr>
<tr>
<td>Plasma glutathione peroxidase</td>
<td>-0.42</td>
<td>0.001</td>
</tr>
<tr>
<td>Plasma glutathione peroxidase</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Erythrocyte glutathione peroxidase</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

\( ^1 \text{HbA1c, glycated hemoglobin, expressed as a percentage of total hemoglobin.} \)

\( ^2 \text{Adjusted for effect of chronic pancreatitis.} \)
Lipids and LDL composition

The mean values for fasting plasma concentrations of lipid, lipoprotein, and apo B are reported in Table 6. Average total cholesterol, LDL-cholesterol, and apo B concentrations were significantly lower in the CP subjects than in the control nondiabetic and diabetic groups. These 3 variables were also significantly lower in diabetic than in nondiabetic subjects (P < 0.01). No significant differences in HDL-cholesterol or triacylglycerol concentrations were observed between groups. An analysis of the plasma LDL composition found significantly (% by wt of total fatty acids) higher concentrations of triacylglycerol in CP patients than in the control diabetic and nondiabetic subjects.

BMI was correlated with LDL cholesterol in the whole population of CP patients, even after adjustment for the effect of diabetes (r = 0.35, P = 0.014; n = 47), and in the CP diabetic group (r = 0.42, P = 0.021; n = 35). Biliary acid content of the stool was significantly higher in CP patients than in control subjects (1446 ± 1054 and 386 ± 208 μmol/24 h, respectively; P = 0.041) and was significantly correlated with the amount of steatorrhea (r = 0.61, P = 0.007) in the entire population of CP patients.

LDL composition of fatty acids

Table 6 lists the mean (±SD) percentag of the LDL fatty acid content. Patients with CP had mean percentages of monounsaturated fatty acids (MUFAs) that were significantly higher than those in the control diabetic or control nondiabetic subjects (P < 0.0001). However, no relation with steatorrhea, BMI, or LDL cholesterol was found. These high MUFA percentages were associated with a slightly but significantly lower polyunsaturated fatty acid content in CP patients (P < 0.01). There was an inverse and significant correlation between MUFA percentages and α-tocopherol concentrations (r = 0.46, P < 0.001) in LDL for the entire population of subjects.

Susceptibility of LDL to oxidation

Two-way ANOVA showed that the lag phase and LDL oxidation were lower in CP subjects with or without diabetes (Table 7), whereas there was no significant effect of DM. However, the lag phase and LDL oxidation were significantly lower in CP nondiabetics than in CP diabetics (DM × CP interaction and Scheffe’s F test: P < 0.05). For the population as a whole (n = 65), univariate regression analyses showed that the lag phase in LDL oxidation was positively correlated with the LDL vitamin E concentrations (r = 0.49, P < 0.001; Figure 1) and plasma concentrations of β-carotene (r = 0.49, P < 0.001) and negatively correlated with the fasting plasma concentration of glucose (r = −0.34, P = 0.006). The lag phase was also positively correlated with LDL vitamin E concentrations in each subgroup of diabetic patients (control diabetic group: r = 0.48; P = 0.043; CP diabetic group: r = 0.40; P = 0.053), whereas the correlation with HbA1c was not significant. Lag phase was not correlated with the concentration of any fatty acid or that of any component of LDL, such as cholesterol, triacylglycerol, phospholipids, or apo B. The LDL oxidation rate was not significantly correlated with any antioxidant factor or HbA1c, but it was weakly and negatively correlated with the fasting plasma concentration of glucose (r = −0.23, P = 0.059) and negatively correlated with the MUFA concentration (r = −0.32, P = 0.014).

In a multivariate analysis, lag phase was negatively correlated with fasting plasma concentrations of glucose after adjustment for LDL vitamin E concentration (glucose: r = −0.27, P = 0.015; LDL vitamin E: r = 0.44, P < 0.0001). This analysis

### Table 6

Lipsids; LDL composition in cholesterol, triacylglycerol phospholipid, and apolipoprotein (apo) B; and fatty acid composition

<table>
<thead>
<tr>
<th>Subject group</th>
<th>Control nondiabetic (n = 20)</th>
<th>Control diabetic (type 1 DM) (n = 25)</th>
<th>CP nondiabetic (n = 12)</th>
<th>CP diabetic (n = 35)</th>
<th>Diabetics effect</th>
<th>Chronic pancreatitis effect</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipids (mg/DL)</td>
<td>Total cholesterol</td>
<td>214 ± 23</td>
<td>194 ± 28</td>
<td>192 ± 35</td>
<td>157 ± 30</td>
<td>0.001</td>
<td>0.0001</td>
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<tr>
<td></td>
<td>Triglyceride</td>
<td>115 ± 43</td>
<td>101 ± 39</td>
<td>136 ± 43</td>
<td>107 ± 51</td>
<td>0.042</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>LDL cholesterol</td>
<td>144 ± 23</td>
<td>131 ± 24</td>
<td>115 ± 25</td>
<td>94 ± 23</td>
<td>0.002</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>HDL cholesterol</td>
<td>41 ± 17</td>
<td>46 ± 11</td>
<td>51 ± 16</td>
<td>40 ± 13</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Apo B</td>
<td>120 ± 19</td>
<td>107 ± 26</td>
<td>103 ± 19</td>
<td>85 ± 25</td>
<td>0.004</td>
<td>0.0003</td>
</tr>
<tr>
<td>LDL composition (%)</td>
<td>Cholesterol</td>
<td>39.8 ± 1.6</td>
<td>39.9 ± 1.1</td>
<td>40.0 ± 1.9</td>
<td>38.9 ± 1.69</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td></td>
<td>Triglyceride</td>
<td>5.8 ± 1.7</td>
<td>6.3 ± 1.4</td>
<td>6.5 ± 1.5</td>
<td>7.7 ± 2.0</td>
<td>0.039</td>
<td>0.011</td>
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<td>Phospholipid</td>
<td>27.2 ± 1.1</td>
<td>27.3 ± 1.2</td>
<td>26.7 ± 3.7</td>
<td>27.0 ± 0.9</td>
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<td>NS</td>
</tr>
<tr>
<td></td>
<td>Apo B</td>
<td>27.2 ± 0.9</td>
<td>26.4 ± 1.8</td>
<td>26.8 ± 2.03</td>
<td>26.4 ± 1.1</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>LDL fatty acid (% by wt of total fatty acids)</td>
<td>Saturated</td>
<td>42.3 ± 4.5</td>
<td>42.0 ± 3.9</td>
<td>43.0 ± 3.6</td>
<td>40.1 ± 4.0</td>
<td>NS</td>
<td>NS</td>
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<tr>
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<td>Monounsaturated</td>
<td>16.9 ± 2.5</td>
<td>17.3 ± 2.9</td>
<td>20.1 ± 1.8</td>
<td>20.8 ± 3.2</td>
<td>NS</td>
<td>&lt;0.0001</td>
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<tr>
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<td>Polyunsaturated</td>
<td>40.8 ± 4.1</td>
<td>40.6 ± 3.3</td>
<td>36.9 ± 3.7</td>
<td>39.1 ± 4.9</td>
<td>NS</td>
<td>0.007</td>
</tr>
<tr>
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<td>n-3</td>
<td>1.7 ± 1.1</td>
<td>1.3 ± 0.7</td>
<td>1.4 ± 0.9</td>
<td>1.0 ± 0.7</td>
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</tr>
<tr>
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<td>n-6</td>
<td>39.1 ± 4.5</td>
<td>39.4 ± 3.5</td>
<td>35.3 ± 4.0</td>
<td>38.1 ± 5.2</td>
<td>NS</td>
<td>0.017</td>
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</table>

1 CP nondiabetic, chronic pancreatitis without diabetes; CP diabetic, CP with insulin-treated diabetes; DM, diabetes mellitus.
2 ANOVA 2 × 2 with Scheffe’s test if the interaction was significant.
3 ± SD (all such values).
4 Significantly different from the other 3 groups, P < 0.01.
showed that concentrations of LDL vitamin E and fasting glucose (in plasma) were the main determinants of the lag phase ($R^2 = 0.32$). After adjustment for LDL vitamin E concentrations, β-carotene was not significantly correlated with the lag phase, but concentrations of those 2 antioxidants were highly correlated ($r = 0.61, P < 0.001$).

**Malondialdehyde-modified LDL autoantibodies and anti-malondialdehyde immune complexes**

MDA-LDL autoantibody concentrations did not differ significantly among the 4 groups (CP nondiabetic: 5.04 ± 2.17; CP diabetic: 4.38 ± 1.46; control nondiabetic: 5.47 ± 2.60 arbitrary units). Anti-MDA-IC concentrations also did not differ significantly among the 4 groups (CP nondiabetic: 1.18 ± 0.28; CP diabetic: 1.35 ± 0.50; control diabetic: 1.30 ± 0.61; control nondiabetic: 1.18 ± 0.34 arbitrary units). However, anti-MDA-ICs were significantly higher in patients with retinopathy ($n = 22, P = 0.014$) or with macroangiopathy ($n = 13; P < 0.001$) than in those without those conditions.

**DISCUSSION**

This study shows that antioxidant status is highly altered and that LDL resistance to oxidation is significantly impaired in patients with CP. A low LDL vitamin E concentration was the main variable that could explain the increase in LDL oxidizability. A high fasting glucose concentration also influences LDL oxidizability, whereas the MUFA content in LDL was associated with a lower oxidation rate. The concentration of anti-MDA-ICs was higher in diabetic patients with retinopathy or macroangiopathy (or both).

The lag phase is usually poorly correlated with the vitamin E concentration in healthy subjects (18, 46–49), probably because of the small range of LDL α-tocopherol values. The wide range of LDL α-tocopherol values in CP patients with fat malabsorption, however, gives rise to a highly significant correlation between these 2 variables. The effect of vitamin E deficiency on LDL oxidation was previously analyzed in young (39-45 y) cystic fibrosis patients (50). We confirm those findings here in older (39-45 y) CP patients with DM. These older patients showed signs of advanced atherosclerosis, whereas the younger patients studied by Winklhofer et al (50) did not show extensive atherosclerotic development.

The low LDL-cholesterol and apo B concentrations in CP patients with diabetes had no influence on LDL oxidizability. This finding is in agreement with 2 studies in which the effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors on this variable were analyzed, except for the 3-hydroxy-2-methylglutaryl coenzyme A reductase inhibitor fluvastatine, which could have an antioxidant effect of its own (51, 52). Conjugated dienes are formed when polyunsaturated fatty acids react with hydroxyl radicals. Consequently, as we show here, the increase in the MUFA content is associated with a slowing of the oxidation process. This protective effect of MUFA on LDL oxidation is also due to their antioxidant properties.

**TABLE 7**

<table>
<thead>
<tr>
<th>Subject group</th>
<th>Control nondiabetic</th>
<th>Control diabetic (type 2 DM)</th>
<th>CP nondiabetic</th>
<th>CP diabetic</th>
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<tbody>
<tr>
<td><strong>Lag phase (min)</strong></td>
<td>104.9 ± 14.6a</td>
<td>90.4 ± 27.1</td>
<td>75.7 ± 19.3b</td>
<td>87.3 ± 20.84c,d</td>
</tr>
<tr>
<td><strong>Propagation (nmol diene · min⁻¹ · mg⁻¹)</strong></td>
<td>5.6 ± 1.18</td>
<td>5.02 ± 1.1</td>
<td>3.8 ± 0.81e</td>
<td>4.2 ± 0.98f,g</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>$P^2$</th>
<th>Diabetes effect</th>
<th>Chronic pancreatitis effect</th>
<th>Interaction</th>
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<td>NS</td>
<td>0.007</td>
<td>0.030</td>
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</tr>
<tr>
<td>NS</td>
<td>0.0002</td>
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</table>

1 CP nondiabetic, chronic pancreatitis without diabetes; CP diabetic, CP with insulin-treated diabetes; DM, diabetes mellitus.

2 ANOVA 2 × 2 with Scheffe’s test if the interaction was significant.

3 $\bar{x} \pm$ SD (all such values).

4 Significantly different from CP nondiabetic group, $P < 0.05$.

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

$^a$ Significant effect Interaction, $P < 0.05$.
oxidizability and on atherosclerosis is well known (53). However, in the current study, the effect was not sufficient to enhance the lag phase and to protect the vitamin E–deficient LDL from oxidation. An inverse and significant correlation between LDL MUFA and LDL α-tocopherol concentrations was identified here, which could lead to an underestimation of the effect of MUFA on LDL oxidation.

We also found a significant and independent correlation between the fasting plasma concentration of glucose and the lag phase duration. Glucose can generate the oxygen radical O$_2^–$ in the presence of transition metal cations, and glycation products may also generate radicals that cause lipid peroxidation. In addition, some antioxidant enzymes may be altered by hyperglycemia. We found a significant negative correlation between HbA1c and glutathione peroxidase activity, which suggested that this enzyme could be glycated (54, 55). However, the antioxidant enzyme activities measured here did not differ significantly between the diabetic and nondiabetic patients, which is in agreement with the findings reported by Akkus et al (56). In contrast, some reports have described a decrease in the activities of erythrocyte SOD (57), catalase (58), and glutathione peroxidase (59). We can explain this result by the relatively good glycemic control (HbA1c) in diabetic patients in our study.

In view of the findings presented here, it seems clear that DM alters the antioxidant status and that this condition increases the risk of antioxidant deficiencies in CP. However, it is also possible that CP patients with weak antioxidant defenses are prone to diabetes.

Oxidized LDL can trigger an autoimmune response that leads to the formation of antibodies (60). Autoantibodies to epitopes of oxidized LDL were shown in the sera of normal and Watanabe hyperlipidemic rabbits and in humans (60, 61) and were reported as an independent predictor of the progression of carotid atherosclerosis in Finnish men (61). In the same way, ICs were shown in patients with coronary disease and hyperlipidemia and in normal subjects. However, there are conflicting reports in the literature concerning the titration of antibodies to oxidized LDL and LDL–ICs (39, 62, 63). Contrary to the findings of a previous study (64), the ratio of oxidized LDL autoantibodies measured here was not significantly higher in the control diabetic group than in the other groups. In fact, Festa et al (64) reported the highest ratio in control diabetic patients, whereas patients with a high microangiopathy score had a lower antibody ratio than did patients without complications. The ratio of oxidized LDL autoantibodies in CP patients has never been reported. We found a higher concentration of oxidized LDL–ICs, which has been proposed to mask free autoantibodies (64), in control diabetic and CP diabetic subjects with microangiopathy or macroangiopathy than in those without. It has been reported that the induction of foam cell formation in vitro is more efficient when the incubation is carried out in the presence of LDL–ICs.

In agreement with other studies (65, 66), we observed a positive correlation between LDL cholesterol and BMI. The low LDL–cholesterol concentration can be mainly explained by the malabsorption of biliary acids, although the malabsorption of cholesterol is an additional factor that could contribute to the low LDL–cholesterol concentration (67). The ratio of polyunsaturated to saturated fats in the diet did not differ significantly among the 4 groups (data not shown). Consequently, LDL cholesterol appears to be a good marker of pancreatic insufficiency and a strong indicator of the nutritional status of CP patients.

A limitation of this study is that the association between vitamin E deficiency and an increase in LDL oxidizability does not indicate a causal relation. The possibility that both vitamin E deficiency and LDL oxidizability are under the influence of a common factor should be considered.

In conclusion, this study shows that antioxidant status is altered in CP patients. The perturbations are particularly pronounced in subjects with CP plus DM. Lower LDL vitamin E concentrations are associated with a significantly higher LDL oxidizability. We have yet to ascertain whether the differences in antioxidant status found in this study are simply the consequence of CP, DM, or both, or whether these differences in turn, contribute to the clinical expression of the diseases. Moreover, the efficacy of an antioxidant supplement, particularly that of a vitamin antioxidant, has yet to be studied.

We thank JM Virion (Centre d’Investigation Clinique, CHU-INSERM, Hôpital Jeanne d’Arc, Toul, France) for help with the statistical analysis. DQ had responsibility for the conception and organization of the study, analysis of the results, subject recruitment, and writing. EW had responsibility for laboratory analysis and analysis of the results. JPB had responsibility for fatty acids analysis. JCF had responsibility for the conceptual basis of the study, laboratory management, analysis of the results, and writing the manuscript. OZ had responsibility for clinical department management, the conceptual basis of the study, and analysis of the results. None of the authors had any personal or financial conflict of interest.

REFERENCES

Relation between liver fat content and the rate of VLDL apolipoprotein B-100 synthesis in children with protein-energy malnutrition\textsuperscript{1–3}

Asha Badaloo, Marvin Reid, Deanne Soares, Terrence Forrester, and Farook Jahoor

ABSTRACT

Background: Fatty infiltration of the liver is associated with an increased morbidity and mortality in children with severe protein-energy malnutrition (PEM), but its pathogenesis remains unclear. Although impaired synthesis of VLDL apolipoprotein B-100 (VLDL-apo B-100) is generally accepted as the pathogenetic mechanism, the rate of it synthesis has not been measured in children with PEM.

Objective: The objective of the study was to ascertain the relation between the degree of hepatic steatosis and the rate of VLDL-apo B-100 synthesis in children with PEM.

Design: The fractional and absolute rates of VLDL-apo B-100 synthesis were measured with a prime-constant intravenous infusion of [\textsuperscript{2}H\textsubscript{3}]leucine in 13 severely malnourished children (8 boys and 5 girls) aged 7–18 mo. Hepatic fat content was estimated by computerized tomography scanning by using the ratio of liver to spleen (L:S) attenuation. The ratio is inversely related to hepatic fat content such that the lower the L:S, the greater the amount of fat in the liver.

Results: There were significant inverse relations between L:S attenuation and VLDL-apo B-100 concentration (\(P < 0.02\)), the absolute rate of VLDL-apo B-100 synthesis (\(P < 0.02\)), and plasma triacylglycerol (\(P < 0.02\)) and serum cholesterol (\(P < 0.05\)) concentrations.

Conclusions: These results suggest that children with PEM synthesize VLDL-apo B-100 at a faster rate as the degree of hepatic fat infiltration increases. Thus, fatty infiltration of the liver in PEM is not due to a reduction in the synthesis of VLDL-apo B-100. Am J Clin Nutr 2005;81:1126–32.

KEY WORDS VLDL apolipoprotein B-100, VLDL-apo B-100, protein-energy malnutrition, children, fatty liver

INTRODUCTION

Fatty liver is a common feature of children with protein-energy malnutrition (PEM), and a hepatic lipid content \(>40\%\) of liver weight is associated with a very poor prognosis (1, 2). Although it is generally accepted that impaired synthesis and secretion of VLDL apolipoprotein B-100 (VLDL-apo B-100) is the primary underlying defect responsible for the excess triacylglycerol deposition in the livers of these children (2–6), there has never been any direct experimental evidence to support this understanding. The precise mechanism of the excess triacylglycerol deposition has therefore remained a topic of debate (7–9).

The main component of VLDL is triacylglycerol, but the lipoprotein also transports cholesterol, cholesterol esters, and phospholipids from the liver to other tissues of the body. The first proposal that impaired synthesis of the apolipoprotein moiety of VLDL was responsible for the excess deposition of triacylglycerol in the livers of malnourished children was based exclusively on clinical observations that children with PEM and fatty livers had lower serum \(\beta\)-lipoprotein (ie, LDL) cholesterol and triacylglycerol concentrations at admission than during nutritional rehabilitation, when liver fat content was believed to start receding (4, 5, 9), and also in comparison with the values in well-nourished children (5). This, however, was not a consistent observation (6, 10–12). It was further suggested that the impaired synthesis of VLDL-apo B-100 was due to a shortage of amino acids because of the chronically inadequate dietary protein intake of children with severe malnutrition (2, 6, 9, 13). The argument for this proposal was based on observations of a slower rate of incorporation of radiolabeled glycine into the protein moiety of LDL and VLDL in rats fed a low-protein diet (13) and of the greater amount of radiolabeled oleic acid incorporated into plasma triacylglycerol after rats fed a protein-free diet were injected with a plasma protein fraction containing the apoprotein of LDL (13). However, the dietary protein provided to the rats in both studies was not comparable to the amount provided by any human diet, even including the poor diets that precipitate PEM. Although the evidence is not conclusive, to date the rate of VLDL-apo B-100 synthesis has not been measured directly in malnourished children. In the current study, we used a stable isotope tracer method to measure the rate of VLDL-apo B-100 synthesis in severely malnourished children who were receiving

\textsuperscript{1} From the Tropical Metabolism Research Unit (AB, MR, and TF) and the Section of Radiology (DS), University Hospital of the West Indies, University of the West Indies, Mona, Kingston, Jamaica, and the US Department of Agriculture/Agricultural Research Service, Department of Pediatrics, Children’s Nutrition Research Center, Baylor College of Medicine, Houston, TX (FJ).

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\textsuperscript{3} Reprints not available. Address correspondence to F Jahoor, Department of Pediatrics, Children’s Nutrition Research Center, Baylor College of Medicine, 1100 Bates Street, Houston, TX 77030-2600. E-mail: fjahoor@bcm.tmc.edu. Received September 10, 2004. Accepted for publication January 10, 2005.
TABLE 1
Diagnosis and anthropometric characteristics of the children at time of study.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Diagnosis</th>
<th>Sex</th>
<th>Age (mo)</th>
<th>Weight (kg)</th>
<th>Length (cm)</th>
<th>Weight-for-age</th>
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1 MK, marasmic kwashiorkor; M, marasmus; K, kwashiorkor.
2 Percentage of the expected median value for a child of the same age or the same length (35).

a 5% dextrose solution. We also investigated the relation between the rate of VLDL-apo B-100 synthesis and liver fat content as ascertained by computerized tomography (CT) scanning (14–16). Dietary protein was not given because we believed that it would not be easy to disentangle any possible independent effect of providing amino acids on the rate of VLDL-apo B-100 synthesis.

SUBJECTS AND METHODS

Subjects

Thirteen children were recruited from among those admitted to the Tropical Metabolism Research Unit (TMRU) of the University of the West Indies for treatment of severe malnutrition. There were 8 boys and 5 girls aged 7–18 mo who were diagnosed with kwashiorkor, marasmic kwashiorkor, and marasmus according to the Wellcome Classification (17). The physical characteristics of the subjects are shown in Table 1. Written informed consent was obtained from at least one parent of each child enrolled. The Faculty of Medical Sciences/University Hospital of the West Indies Ethics Committee and the Baylor Affiliates Review Board for Human Subject Research, University of the West Indies for treatment of severe malnutrition.

Resuscitative treatment

During hospitalization, the children were treated according to a standard treatment protocol that is based on an understanding of the metabolic state at different stages of treatment (18). Briefly, the initial phase was a resuscitative period of treatment from admission until appetite returned, edema was lost, and infection was cleared. During this period, fluid and electrolyte imbalances were corrected, infections were treated with broad-spectrum antibiotics, and the children were fed a milk (Nan; Nestlé SA, Vevey, Switzerland)-based resuscitative diet (protein: 5% of energy; carbohydrate: 74% of energy; fat: 21% of energy) that aimed to provide ≈4.17 kJ · kg⁻¹ · d⁻¹ and ≈1.2 g · kg⁻¹ · d⁻¹ of protein, which is adequate for maintenance of body weight. Feeds were offered as boluses every 3 h or as smaller boluses every 2 h if the child was having problems tolerating the larger volume of formula. The diet was supplemented with vitamins and a mineral mix to provide adequate amounts of micronutrients. This study was carried out during the acute resuscitation phase, but, on the day of the study, the subjects did not receive the usual milk feeds during measurements (see Study design).

Study design

The rate of VLDL-apo B-100 synthesis was measured with an intravenous infusion of isotopically labeled leucine a mean (±SEM) 4 ± 1 d after admission. The isotope infusions were performed over a 6-h period, starting 2 h after the last bolus meal. To avoid hypoglycemia during the infusion protocol, a 5% dextrose solution was infused intravenously at 3 mg · kg⁻¹ · min⁻¹ starting 2 h before the isotope infusion. A noncontrast abdominal CT scan was performed within 24 to 48 h of the isotope infusion.

Assessment of liver fat

Assessment of liver fat content was done by CT scan on the basis of previously established criteria that have been validated by liver biopsy and liver histomorphometry (14–16). Enrolled children underwent a noncontrast abdominal CT scan within 24 to 48 h of the infusion by using a Sytecsynergy scanner (General Electric Co, Fairfield, CT). The children were sedated with chloral hydrate (50 mg/kg) before each scan according to standard radiologic practice. To reduce radiation exposure, a single cross-sectional CT scan of 10-mm thickness was taken at the level of the intervertebral disc between the body of the 12th thoracic and 1st lumbar vertebrae to include both lobes of the liver, the renal cortex, and the spleen. For all scans, the window level and window width were kept constant, and the machine was operated in the tissue optimization mode. A region of interest (ROI) was placed on 4 areas of the liver and 1 area of the spleen: at depths of 1.5, 2.0, and 3.0 cm from the liver capsule on the right lobe of the liver, at a depth of 1.5 cm from the liver capsule on the left lobe, and at a depth of 1.5 cm from the splenic capsule on the spleen. Splenic attenuation was measured from the latter ROI. Care was taken not to include major portal, arterial, and venous
vessels. For each ROI, the attenuation measured in Hounsfield units (HU) was recorded. The mean liver attenuation was calculated from the 4 liver ROIs, and the ratio of mean liver attenuation to spleen attenuation (L:S) was ascertained. L:S is inversely related to hepatic fat content such that the lower the L:S, the greater the amount of fat in the liver, and a ratio <1 denotes significant hepatic steatosis (16, 19, 20).

Tracer infusion protocol

The rate of VLDL-apo B-100 synthesis was measured over a 6-h period by intravenous infusion of a sterile solution of $[^2H]_3$leucine (Cambridge Isotope Laboratories, Woburn, MA). Two intravenous access sites were established in opposite arms by the insertion of 22- or 24-gauge catheters after preparation of the access sites with a topical anesthetic (EMLA cream; Astra Pharmaceuticals Ltd, Langley, United Kingdom). One intravenous catheter was used for infusion of the $[^2H]_3$leucine and the other for blood sampling. After a 2-mL venous blood sample was stored immediately at $-70°C$ for later analyses. Serum cholesterol was assayed with the use of the enzymes cholesterol esterase and cholesterol oxidase (Infinity cholesterol reagent; Sigma Diagnostics Inc, St Louis, MO). Plasma triacylglycerol concentration was measured by radial immunodiffusion on an aliquot of the supernatant. The amino acids released from the protein by acid hydrolysis were purified by cation exchange chromatography and converted to the $n$-propyl ester heptaflourobutyramide derivative, and leucine isotopic enrichment was measured by gas chromatography–mass spectrometry as described (22). Briefly, the amino acids released from the protein by acid hydrolysis were purified by cation exchange chromatography and converted to the $n$-propyl ester heptaflourobutyramide derivative, and leucine isotopic ratio was measured by monitoring ions at a mass-to-charge ratio of 349 to 352 on a gas chromatography mass spectrometer (model 5988A; Hewlett-Packard, Palo Alto, CA). VLDL-apo B-100 concentration was measured by radial immunodiffusion on an aliquot of the supernatant.

Calculations

The fractional synthesis rate (FSR) of VLDL-apo B-100 was calculated from the rate of incorporation of $[^2H]_3$leucine into the protein during the rise to a plateau and the isotopic enrichment of the protein at the plateau, as described by Lichtenstein et al (23).

$$\text{FSR}(%/h) = \left[ (\text{IE}_{t_2} - \text{IE}_{t_1}) \times 100 / \text{IE}_{\text{pool}} \times t_2 - t_1 \right]$$  

where $\text{IE}_{t_2} - \text{IE}_{t_1}$ is the rate of increase in isotopic enrichment of apo B-100–bound leucine from time $t_1$ to $t_2$ during the rise to a plateau, and $\text{IE}_{\text{pool}}$ is the isotopic enrichment of apo B-100–bound leucine at the plateau. A nonlinear curve fit was performed with PRISM software (version 3; GraphPad Software, San Diego, CA) using a one-phase exponential equation. Only points that fell on the linear portion of the curve during the rise to the plateau were used to calculate the rate of incorporation of tracer into the protein. The plateau isotopic enrichment of apo B-100–bound leucine in plasma was assumed to represent the isotopic enrichment of the intrahepatic leucine pool (precursor pool) from which the VLDL-apo B-100 is synthesized (22–24).

The intravascular absolute synthesis rate (ASR) of VLDL-apo B-100 was estimated as the product of the FSR and the intravascular VLDL-apo B-100 mass, which itself is the product of the plasma volume (specific for diagnosis) and the plasma concentration of VLDL-apo B-100. The plasma volume was based on measurements that we performed in a group of malnourished children during the early resuscitative phase. In these plasma volume experiments, we improved on the method of Gibson and Evans (25) by taking multiple postinjection samples to account for the possible confounding effects of changes in the transcapillary escape rate of albumin-bound Evans blue. By using semi-logarithmic extrapolation to time zero, we obtained mean plasma volume estimate of 65, 76.9, and 75.8 mL/kg in patients with kwashiorkor ($n = 14$), marasmic kwashiorkor ($n = 10$), and marasmus ($n = 9$), respectively.

Statistical analysis

The associations between L:S and both VLDL-apo B-100 kinetics and plasma lipid concentration were ascertained by regression analyses. Data analysis was performed by using STATA for WINDOWS statistical software (version 8; Stata Corp, College Station, TX).

RESULTS

Thirteen children, aged 7–18 mo, participated in the study. On the basis of the Wellcome Classification (17), 6 were diagnosed with marasmus, 3 with kwashiorkor, and 4 with marasmic-kwashiorkor. The physical characteristics of the subjects are presented in Table 1. All of the subjects were severely malnourished and had markedly lower than expected weight-for-age and weight-for-length. Biochemical measurements in the subjects are shown in Table 2. All subjects were anemic, and 11 were hypoalbuminemic. Plasma bilirubin concentrations were within the normal range in all of the subjects. Aspartate aminotransferase concentration, measured in 12 of the children, was above normal. Eleven of the 13 subjects had evidence of one or more infections at admission.

The actual mean intakes of energy and protein before the isotope infusion protocol were $\approx 89\%$ of the goal, which reflects the anorexia that is characteristic of the early resuscitative phase of treatment (Table 3). There were no significant differences between mean protein or energy intakes before the experiment and VLDL-apo B-100 concentration, FSR, or ASR. Liver span, the liver and spleen CT attenuation numbers, and L:S attenuation are shown in Table 4. Five of the children had L:S <1, which indicated marked hepatic steatosis. There were significant inverse relations between L:S and the VLDL-apo B-100 concentration ($P < 0.02; \text{Figure 1}$), the VLDL-apo B-100 ASR ($P < 0.02; \text{Figure 2}$), plasma triacylglycerol concentrations ($P <
DISCUSSION

The aim of this study was to ascertain whether the fatty liver seen in severely malnourished children is related to impaired synthesis of the apo B-100 moiety of VLDL. Our results showed that the children with more fat in their livers (ie, lower L:S) synthesized VLDL-apo B-100 at a faster rate than did children with less fat in their livers, and that this faster synthesis was associated with higher plasma concentrations of the lipoprotein. The children with more fat in their livers also had higher concentrations of plasma triacylglycerol and cholesterol than did the children with less fat in their livers, which suggested increased mobilization of lipids from the liver. These findings suggest that greater lipid deposition in the livers of children with severe malnutrition is not associated with impaired synthesis of VLDL-apo B-100, as was proposed previously (2–6).

In the current study, it was not considered ethical to perform a biopsy of liver tissue. CT scanning has emerged as an excellent noninvasive tool for detecting and quantifying hepatic fat deposition (16, 19, 20), because it shows a significant inverse relation between the hepatic fat content and the hepatic attenuation number. As fat accumulates in the liver, the tissue’s absorption of the X-rays decreases, which leads to a decrease in the CT HU (14–16). The use of absolute CT attenuation to define hepatic steatosis is limited, however, because of variations of the number. This is due to the fact that the actual HU is affected by nonhepatic factors, such as body size and instrument variations (20, 26). Expressing liver attenuation in proportion to splenic attenuation (L:S), however, overcomes this limitation because the spleen

TABLE 2
Biochemical characteristics of the children at hospital admission

<table>
<thead>
<tr>
<th>Subject</th>
<th>Hemoglobin (g/dL)</th>
<th>WBCs (10^3/L)</th>
<th>Glucose (mmol/L)</th>
<th>Albumin (g/L)</th>
<th>AST (IU/L)</th>
<th>Total bilirubin (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.4</td>
<td>8.7</td>
<td>5.7</td>
<td>22</td>
<td>79</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>6.4</td>
<td>9.8</td>
<td>6.2</td>
<td>22</td>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>7.1</td>
<td>9</td>
<td>11.6</td>
<td>24</td>
<td>53</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>8.2</td>
<td>10.8</td>
<td>4.4</td>
<td>34</td>
<td>178</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>8.2</td>
<td>11.6</td>
<td>9</td>
<td>38</td>
<td>ND</td>
<td>4</td>
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<td>6</td>
<td>8.7</td>
<td>8.7</td>
<td>3.3</td>
<td>24</td>
<td>70</td>
<td>2</td>
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<tr>
<td>7</td>
<td>8.8</td>
<td>7.5</td>
<td>4</td>
<td>30</td>
<td>99</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>8.7</td>
<td>7.3</td>
<td>4.8</td>
<td>24</td>
<td>51</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>9.4</td>
<td>9.9</td>
<td>5.5</td>
<td>19</td>
<td>73</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>9.7</td>
<td>6.7</td>
<td>2.9</td>
<td>27</td>
<td>109</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>10.1</td>
<td>11.4</td>
<td>3.6</td>
<td>30</td>
<td>43</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>13.5</td>
<td>8.8</td>
<td>7.1</td>
<td>31</td>
<td>35</td>
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</tr>
<tr>
<td>13</td>
<td>8.4</td>
<td>18.1</td>
<td>6.7</td>
<td>39</td>
<td>44</td>
<td>8</td>
</tr>
<tr>
<td>x</td>
<td>8.8</td>
<td>9.9</td>
<td>5.8</td>
<td>28</td>
<td>72</td>
<td>4.9</td>
</tr>
<tr>
<td>SEM</td>
<td>0.5</td>
<td>0.8</td>
<td>0.7</td>
<td>2</td>
<td>12</td>
<td>0.7</td>
</tr>
</tbody>
</table>

1 WBCs, white blood cells; AST, aspartate aminotransferase; ND, not done. Normal ranges were 12–12.5 g/dL for hemoglobin, 32–50 g/L for albumin, 7–24 IU/L for AST, and <17 μmol/L for total bilirubin.

TABLE 3
Dietary energy and protein intakes of children on the day before isotope infusion

<table>
<thead>
<tr>
<th>Subject</th>
<th>Protein intake (g·kg⁻¹·d⁻¹)</th>
<th>Energy intake (kcal·kg⁻¹·d⁻¹)</th>
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<tbody>
<tr>
<td>1</td>
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</tr>
<tr>
<td>3</td>
<td>1.02</td>
<td>354</td>
</tr>
<tr>
<td>4</td>
<td>0.82</td>
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</tr>
<tr>
<td>5</td>
<td>0.99</td>
<td>346</td>
</tr>
<tr>
<td>6</td>
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<td>7</td>
<td>1.12</td>
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<td>9</td>
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<td>10</td>
<td>1.11</td>
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<td>11</td>
<td>0.73</td>
<td>253</td>
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<tr>
<td>12</td>
<td>1.10</td>
<td>383</td>
</tr>
<tr>
<td>13</td>
<td>1.20</td>
<td>418</td>
</tr>
<tr>
<td>x ± SEM</td>
<td>1.07 ± 0.04</td>
<td>373 ± 14</td>
</tr>
</tbody>
</table>

TABLE 4
Liver span and computerized tomography (CT) scans of liver and spleen of children

<table>
<thead>
<tr>
<th>Subject</th>
<th>Liver span (cm)</th>
<th>Liver CT attenuation (Hounsfield unit)</th>
<th>Spleen CT attenuation (Hounsfield unit)</th>
<th>Liver:spleen attenuation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>10</td>
<td>40</td>
<td>0.25</td>
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<tr>
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<td>4.5</td>
<td>54</td>
<td>45</td>
<td>1.20</td>
</tr>
<tr>
<td>3</td>
<td>8.2</td>
<td>65</td>
<td>40</td>
<td>1.62</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>27</td>
<td>40</td>
<td>0.67</td>
</tr>
<tr>
<td>5</td>
<td>7.5</td>
<td>67</td>
<td>51</td>
<td>1.32</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
<td>44</td>
<td>38</td>
<td>1.17</td>
</tr>
<tr>
<td>7</td>
<td>6.9</td>
<td>66</td>
<td>56</td>
<td>1.17</td>
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<td>8</td>
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<td>49</td>
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<td>1.05</td>
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<td>94</td>
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<td>0.22</td>
</tr>
<tr>
<td>12</td>
<td>ND</td>
<td>52</td>
<td>54</td>
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</tr>
<tr>
<td>13</td>
<td>5</td>
<td>50</td>
<td>50</td>
<td>1.74</td>
</tr>
</tbody>
</table>

1 ND, not done. The CT attenuation number is a measure of the attenuation of a tissue compared with the attenuation of water. Fat has much lower attenuation than does water, and therefore lower CT attenuation and L:S indicate more fatty infiltration.

FIGURE 1. The association between VLDL-apolipoprotein B-100 (VLDL-apo B-100) concentrations and the ratio of liver attenuation to spleen attenuation in children with protein-energy malnutrition. CT, computerized tomography. x slope: −44 ± 12 (r² = 0.53, P < 0.02).
acts as an internal control. The splenic attenuation number is normally 8–10 HU less than the liver attenuation number (27), and therefore L:S < 1 is associated with significant hepatic steatosis (16, 19, 20).

The assembly of VLDL and its secretion from the liver is a two-step process. Initially, hepatic apo B-100 associates with lipids to form dense lipoprotein particles in the rough endoplasmic reticulum, and then it is further lipidated to VLDL, probably in the smooth endoplasmic reticulum (28). The general belief that the fatty liver of children with PEM resulted from impaired synthesis of the apoprotein moiety of VLDL was based largely on 2 lines of indirect evidence. The first was observations that the plasma triacylglycerol and cholesterol concentrations in children with severe malnutrition and fatty livers were low at admission and that they rose during treatment (3–6). Thus, the role of impaired VLDL-apo B-100 synthesis in the pathogenesis of the fatty liver of severely malnourished children was imputed from the assumption that an increase in plasma triacylglycerol and LDL-cholesterol concentrations indicated increased synthesis of all lipoproteins (2). However, this generally accepted hypothesis of impaired VLDL-apo B-100 synthesis was never based on any actual measurement of the rate of synthesis. Moreover, several other studies in children with severe malnutrition have found plasma triacylglycerol concentrations that are low (10, 11, 29), normal (10, 11, 29), or high (10–12, 29) at admission and that rise, remain unchanged, or fall, respectively, during treatment (10, 11, 29). Because of these widely varied values, higher plasma triacylglycerol concentrations during treatment cannot be used as a reliable indicator of impaired VLDL-apo B-100 synthesis before treatment.

The second premise linking the accumulation of fat in the liver with impaired synthesis of VLDL-apo B-100 in severely malnourished children was based on the argument that dietary protein deficiency resulted in a reduction in the rate of synthesis of the apolipoprotein. This argument, in turn, was based primarily on the findings of a slower incorporation of radiolabeled glycine into the apoprotein of LDL and VLDL in rats fed a low-protein diet and of a faster incorporation of radiolabeled oleic acid into plasma triacylglycerol when rats fed a protein-free diet were injected with LDL apolipoprotein (6, 13). However, the protein supplied to the rats in both of those studies was not comparable to the amount supplied by any human diet, including the poor diets that precipitate severe malnutrition. In the current study, the children were treated before the experiment with a resuscitative diet that aimed to provide ≈417 kJ · kg⁻¹ · d⁻¹ and ≈1.2 g · kg⁻¹ · d⁻¹ of protein. The actual intake was ≈11% lower, but the absence of any significant association between the amount of dietary protein and the rate of VLDL-apo B-100 synthesis does not support the hypothesis that dietary protein deficiency was the underlying cause of fat deposition in the livers of children with PEM. In addition, there was no significant association between VLDL-apo B-100 synthesis and the energy intake from the resuscitative diet before the studies, which suggested that it was unlikely that the overall resuscitative diet had an effect on apo B-100 synthesis.

Plasma triacylglycerol concentrations were significantly higher in the children with more liver fat than in those with less liver fat. Similarly, the amount of fat in the liver was positively related to the concentrations of VLDL-apo B-100 and serum cholesterol, which indicated enhanced removal of lipid from the livers of the children with greater degrees of hepatic steatosis. Our current findings seem to support the contention of others (7, 8, 12) that impaired VLDL synthesis is not the cause of excess
triacylglycerol deposition in the livers of malnourished children. It may be argued that the significant regression between L:S and the VLDL-apo B-100 ASR and concentration (Figures 1 and 3) is dependent on 2 or 3 subjects; if they were excluded, then there would be no significant relation. In such a case, the data still would not support the widely held belief that hepatic steatosis in malnourished children is due to impaired synthesis of VLDL-apo B-100. One possible limitation of this study is that the plasma volume specific to each child was not used in the calculation of the absolute rate of synthesis. However, plasma volumes specific for kwashiorkor, marasmic-kwashiorkor, and marasmus were used, and the values were based on measurements that we have done in a group of malnourished children of similar age, at a similar stage of rehabilitation, and with similar fluid intake.

There is alternative convincing evidence to support the involvement of other mechanisms in the pathogenesis of the fatty liver of childhood malnutrition (7, 30–33). In theory, the amount of hepatic triacylglycerol available for export can exceed the rate of removal because of 1 or more of 4 possible mechanisms—either increased hepatic fatty acid (FA) synthesis; impaired FA oxidation by hepatocytes; increased hepatic FA influx secondary to a stimulated rate of lipolysis, impaired whole-body FA oxidation, or both; or impaired removal of triacylglycerol from the liver by VLDL. For example, Fletcher (30) proposed that hepatic FA availability was increased because of increased FA synthesis from glucose. This proposal was based on Fletcher’s finding of a significantly lower glucose-6-phosphatase activity in the liver tissue of malnourished children with fatty livers than in the liver tissue of children who had recovered from malnutrition (30). In addition, 2 studies by Lewis et al (31, 32) reported faster plasma palmitate flux and higher FA concentrations, which were indicative of a stimulated rate of lipolysis, in malnourished children with fatty livers than in well-nourished children. An increased lipolysis together with the finding that whole-body lipid oxidation is markedly slower in children with kwashiorkor than in well-nourished children (33) will result in an increased influx of free FA into the liver. Furthermore, decreased peroxisomal β-oxidation of FA by the liver has been proposed as one of the mechanisms of the pathogenesis of fatty liver, on the basis of the markedly lower concentration of peroxisomes in the liver of severely malnourished children at autopsy than in the livers of children who had recovered from malnutrition (7). It is therefore possible that the fatty liver of the malnourished child can result from an increased availability of FA for hepatic reesterification to triacylglycerol and not from impaired VLDL-apo B-100 synthesis.

In conclusion, the findings of this study indicated that synthesis of the apolipoprotein moiety of VLDL in severely malnourished children with more liver fat is not impaired relative to the faster rate of synthesis of apo B-100 in the children with more liver fat. This occurs even in the face of the slower rate of whole-body protein turnover that is characteristic of the severely malnourished state (34).

We are grateful to the physicians and nursing staff of the Tropical Metabolism Research Unit for their care of the children and to Hyacinth Gallimore, Bentley Chambers, Sharon Howell, Margaret Frazer, and Melanie Del Rosario for their excellent work and support in the conduct of the studies and analysis of the samples.

AB contributed to all aspects of the production of this manuscript: the design of the study, execution of the experiments, data collection, analysis and interpretation, and writing of the manuscript. MR and TF were involved in the design of the study, analysis and interpretation of the data, and writing of the manuscript. DS shared responsibility with MR for CT scan measurement and was also involved in writing the manuscript. FJ was involved in the design of the study, analysis and interpretation of the data, and writing of the manuscript. None of the authors had any personal or financial conflict of interest.

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A controlled 2-mo dietary fat reduction and soy food supplementation study in postmenopausal women1–3

Anna H Wu, Frank Z Stanczyk, Carmen Martinez, Chiu-Chen Tseng, Suzanne Hendrich, Patricia Murphy, Sukanya Chaikittisilpa, Daniel O Stram, and Malcolm C Pike

ABSTRACT

**Background:** Low intake of dietary fat and high intake of soy foods have been suggested to partly explain the lower breast cancer rates in Asia, perhaps because of lower endogenous estrogens.

**Objective:** The objective was to assess the hormonal and nonhormonal effects of diets resembling an Asian diet in terms of total fat and soy food contents.

**Design:** Fifty-seven postmenopausal women participated in a randomized, controlled, dietary intervention study. The subjects consumed a very-low-fat diet (VLFD; 11% of energy as fat), a Step I diet (25% of energy as fat) supplemented with soy food (SFD; 50 mg isoflavones/d), or a control Step I diet (CD; 27% of energy as fat) with no soy food. All diets were prepared at the General Clinical Research Center of the University of Southern California. Serum hormones and other markers were measured at baseline and every 2 wk during the 8 wk of intervention.

**Results:** There were no significant differences in total estradiol and sex hormone binding globulin at the completion of the intervention between women in the SFD and VLFD groups and those in the CD group. Serum insulin decreased significantly in the SFD group, and leptin decreased significantly in the SFD and VLFD groups; however, these changes did not differ significantly from the changes in the CD group.

**Conclusions:** This study does not provide evidence that ingestion of soy food or a VLFD significantly reduces estrogen concentrations in postmenopausal women. However, short-term changes in diet may have significant and beneficial effects on blood insulin and leptin concentrations.

KEY WORDS Controlled randomized trial, low-fat diet, soy food, blood biomarkers

INTRODUCTION

The role of diet in the etiology of breast cancer remains controversial in observational epidemiologic studies. Pooled analyses of cohort studies conducted in Western countries, which used food-frequency questionnaires, showed no relation between fat intake and breast cancer risk (1). However, in the Norfolk component of the European Prospective Investigation of Cancer study, which used a 7-d food diary, risk of breast cancer increased significantly with increasing intakes of total and saturated fat (2). These differences in cohort findings emphasize the importance of measurement error in dietary assessment (3). The role of soy in the etiology of breast cancer is also inconclusive. Breast cancer risk was unrelated to soy intake in studies conducted in Western populations, where intake was low (median daily intake <1 mg soy isoflavones) (4, 5). Although breast cancer risk was significantly inversely associated with soy intake in several studies conducted in Asian populations with substantial soy intakes, this finding remained after adjustment for dietary and nondietary risk factors (6, 7), the possibility of residual confounding cannot be ruled out for certain. Because of findings of stimulatory effects of dietary genistein (a main isoflavone in soybeans) in human breast (8) and breast cancer cell growth in MCF-7 cells and in an ovariectomized athymic mice model (9), there are also concerns regarding the safety of soy intake, particularly in postmenopausal women.

There is compelling evidence that estrogen concentrations are a critical determinant of breast cancer risk (10). Dietary fat reduction (11) and soy supplementation intervention studies (12) have been conducted with the rationale that a reduction in endogenous estrogen concentrations in short-term settings will lend support to a role of dietary fat or soy on breast cancer risk. However, the quality of these intervention studies, particularly the dietary fat reduction studies, has been questioned (13). To further examine the influence of dietary fat reduction and soy food supplementation on circulating hormone concentrations in postmenopausal women, we conducted a randomized, controlled, dietary intervention study. We investigated the separate effects on hormonal endpoints of a soy food–supplemented Step I diet (30% of energy as fat, 50% of energy as carbohydrate, 20% of energy as protein and designed to provide 50 mg isoflavones/d; SFD) and a very-low-fat, high-carbohydrate diet (designed to provide 12% of energy as fat, 50% of energy as carbohydrate, 20% of energy as protein and designed to provide 50 mg isoflavones/d; SFD) and a very-low-fat, high-carbohydrate diet (designed to provide 12% of energy as fat, 50% of energy as carbohydrate, 20% of energy as protein and designed to provide 50 mg isoflavones/d; SFD) and a very-low-fat, high-carbohydrate diet (designed to provide 12% of energy as fat, 50% of energy as carbohydrate, 20% of energy as protein and designed to provide 50 mg isoflavones/d; SFD) and a very-low-fat, high-carbohydrate diet (designed to provide 12% of energy as fat, 50% of energy as carbohydrate, 20% of energy as protein and designed to provide 50 mg isoflavones/d; SFD).
68% of energy as carbohydrate, 20% of energy as protein, and no soy food; VLFD) compared with a control Step I diet (30% of energy as fat, 50% of energy as carbohydrate, 20% of energy as protein, and no soy food; CD) in free-living postmenopausal women.

SUBJECTS AND METHODS

Subjects

Postmenopausal women were recruited between July 2000 and August 2002 through flyers and newsletters that were distributed on campus and at the University of Southern California (USC) Health Fair, public service announcements on local radio stations, and a one-time advertisement on a local television station. To be eligible for inclusion, subjects had to be postmenopausal (≥1 y since the last menstrual period), ≥50 y of age, and noncurrent users of menopausal hormone therapy (ie, stopped use ≥6 mo before entering study). Women were excluded if they were consuming a special diet (eg, low-fat, high-fiber) or had a history of cancer (other than nonmelanoma skin cancer), diabetes mellitus, or other chronic disease.

A total of 274 women completed an initial telephone screening; 130 were eligible and 70 completed the baseline assessment and consented to be randomly assigned to 1 of 3 dietary arms (see baseline assessment below). Six women withdrew within the first 2 wk of entering the study because they did not wish to adhere to our diet protocol. We excluded another 7 women (1 in the CD group, 5 in the SFD group, and 1 in the VLFD group) because their baseline circulating estrogen concentrations suggested that they were not postmenopausal. The final analysis included 57 women (20 in the CD group, 17 in the SFD group, and 20 in the VLFD group) who represented the racial-ethnic diversity of the study area (17 white, 22 Hispanics, 11 African Americans, and 7 Asians). The study protocol was approved by the USC Institutional Review Board. Written informed consent was obtained from all study participants.

Diets

All the diets in this study were prepared in the Bionutrition Department’s Research Kitchen at the General Clinical Research Center (GCRC) at the Los Angeles County USC Medical Center. Eight-day cycle menus for each of the 3 diets were developed by the nutritionist at the GCRC (see below). During the 8 wk of study, participants received all foods to be consumed from the research kitchen at the GCRC. Each daily menu included foods for breakfast, lunch, dinner, and a morning and an evening snack. The food supply for each week was packed and stored in insulated coolers that the participants took home. Written instructions regarding food safety and reheating methods were provided. Participants were given a daily log to check off all of the foods on the menu that they consumed and to record any extra foods eaten that were not on the menu. They were also asked to return to the kitchen all uneaten food or leftovers in their original containers, which were weighed and deducted from their daily nutrient intake.

The control diet followed the American Dietetic Association guidelines for a healthy balanced diet, including ∼50% of energy from carbohydrates, 30% from fat, and 20% from protein (14). The VLFD, which provided 12% of energy from fat, was developed by using mostly legumes as a source of protein along with fish, lean chicken, and low-fat dairy products. The SFD, which provided 50 mg isoflavones/d (∼15 g soy protein), was developed by using some modified recipes provided by Mori-Nu tofu (Morinaga Nutritional Foods, Torrance, CA) and recipes developed in the GCRC Research Kitchen so that the tofu was disguised in soups, smoothies, sauces, and other dishes. The nutrient contents of the diets were evaluated by using nutritional software (Pronutra version 2.0.1; Viocare, Princeton, NJ, formerly of Princeton Multimedia Technologies). Subjects were assigned to treatment groups at the time of the preentry visit and were blinded to the diet to which they were randomly assigned, and this was largely successful (see below). The isoflavone content of the SFD was monitored throughout the study (see below).

Interested subjects who met the eligibility criteria were mailed a 3-d food record with instructions to record their food intake on 2 typical days of the week and on 1 weekend day. Because the objective of the study was for participants to maintain their body weight during the intervention period, each participant’s caloric requirements were calculated by using the Harris Benedict equation [655 + 4.3 × weight (lb) + 4.3 × height (in) − 4.7 × age (y)]. The value obtained with this equation was multiplied by an activity factor of 1.3 for ambulatory sedentary participants (15). Participants were weighed every 2 wk. If they had lost or gained >2 kg from their initial baseline weight, they were placed on a higher or lower caloric diet as needed. During the 8 wk of intervention, daily nutrient intake was computed based on the daily logs of foods eaten, ie, food provided by the metabolic kitchen, minus foods that were returned, and plus any extra foods consumed. All food records were analyzed by using Pronutra version 2.0.1.

Baseline assessment and data and sample collection

At the initial screening interview, we administered a baseline questionnaire that asked about menstrual, reproductive, and menopausal factors. Body weight, blood pressure measurements, and blood samples were obtained at baseline and after 2, 4, 6, and 8 wk of the intervention. Blood specimens were collected between 0600 and 1100 after the subjects had fasted for 12 h. Serum and plasma were separated by centrifugation (2500 × g, 15 min, 4 °C). On the day of the blood draw, subjects were asked to collect an overnight urine specimen into plastic bottles that contained 1 g ascorbic acid. Urine specimens were separated into 100-mL aliquots and stored at −20 °C.

Daily food records were used as a measure of compliance. In addition, urinary isoflavone concentrations were determined at baseline, at least once during the intervention, and at the completion of the study. Urinary isoflavone concentrations were used as a measure of compliance with the SFD. Although an objective measure of compliance for the CD or the VLFD is not available, we used reductions in HDL cholesterol and increases in triacylglycerol concentrations to confirm a decrease in fat intake and an increase in carbohydrate intake, as was done in other dietary fat reduction trials (16).

Blood hormone, lipid, insulin-like growth factor I, and insulin-like growth factor binding protein 3 and other analyses

Estradiol, estrone, testosterone, and androstenedione were quantified in serum by previously described radioimmunoassay (RIA) methods (17, 18). The intra- and interassay CVs were
between 7% and 16% for estradiol, estrone, and androgens. Free testosterone and free estradiol were calculated on the basis of measured total testosterone and total estradiol concentrations, respectively; sex hormone binding globulin (SHBG) concentrations; and an assumed constant for albumin (19). This method has been found to have high validity ($r = 0.97$) compared with direct measurements (20). Other analytes were measured in serum by highly specific direct immunoassays. SHBG was measured by chemiluminescent immunometric assay on the Immulite analyzer (Diagnostic Products Corporation, Inglewood, CA). The SHBG intra assay and inter assay CVs were 7% and 10%, respectively. A two-site chemiluminescent immunoassay was used to measure insulin-like growth factor I (IGF-I) on the Nichols Advantage Specialty System (Quest Diagnostics Nichols Institute, San Juan Capistrano, CA). The IGF-I intra assay and inter assay CVs were 2% and 10%, respectively. A competitive RIA that used kits obtained from Quest Diagnostics Nichols Institute was used to measure insulin-like growth factor binding protein 3 (IGFBP-3); the intra assay and inter assay CVs were 7% and 12%, respectively. Insulin concentrations were measured by chemiluminescent immunometric assay on the Immulite analyzer (Diagnostic Products Corporation). The intra assay and inter assay CVs were 6% and 8%, respectively. Leptin was quantified by RIA with kits from Linco Research Inc (St Charles, MO); the intra assay and inter assay CVs were 8% and 6%, respectively.

Serum concentrations of lipids and lipoproteins were determined on the Vitros analyzer by using the following methods: total cholesterol was quantified colorimetrically after hydrolysis of cholesterol esters and subsequent oxidation of the free cholesterol. HDL cholesterol was measured colorimetrically after precipitation of LDL cholesterol and VLDL cholesterol by using dextran sulfate and magnesium chloride. Triacylglycerols were also quantified colorimetrically, and LDL cholesterol was calculated as follows:

$$\text{LDL cholesterol} = \text{total cholesterol} - \text{HDL cholesterol} - \text{VLDL-cholesterol (VLDL-cholesterol} = \text{triacylglycerols/5}) \quad (1)$$

**Analysis of isoflavone concentrations in foods and urine**

To determine food and urinary isoflavone concentrations, an HPLC quantitative method was used (21). Urine samples collected at baseline, at the completion of the study, and at least once during the intervention were tested for urinary isoflavone concentrations. Individual soy dishes (fruit smoothies, soups, lasagna, stroganoff, chili, spaghetti and pesto sauces, stir fry, enchiladas, cake, and pies) were homogenized in a food processor, freeze-dried, and analyzed for isoflavone content (all 12 isomers of daidzein, genistein, and glycitein) 8 times during the study to monitor the amount of isoflavones in these foods. The soy food measurements obtained during the study showed that the mean daily intake of isoflavones (as aglycones) was 50.9 mg for those randomly assigned to the soy diet (the range of isoflavones for the 8-d soy menus was 40.3–65.9 mg/d).

**Statistical analysis**

All measurements of interest were transformed logarithmically to achieve approximate normal distributions for statistical analysis. Geometric mean values were computed for urinary isoflavones (genistein, daidzein, and glycitein) and concentrations of serum estrogens (total and free estradiol, estrone), androgens (total and free testosterone, androstenedione), SHBG, lipids (total cholesterol, HDL cholesterol, LDL cholesterol, and triacylglycerols), IGF-1, IGFBP-3, insulin, and leptin. We calculated the daily urine isoflavone excretion as follows:

$$\text{Daily urinary isoflavone} = (\text{isoflavone concentration/creatinine concentration} \times 1.2 \text{~g}) \quad (2)$$

where 1.2 g is the assumed daily creatinine excretion (22). Statistical tests were performed by using the general linear model approach. Student’s $t$ test was used to compare dietary intake, body size, and serum hormone, lipid, and growth factors at baseline between the SFD and CD groups and between the VLFD and CD groups. The main outcome of interest was the relation between diet intervention and the individual differences (ie, week 8 compared with baseline) in the biomarkers of interest; the statistical significance of the possibly different effects of the diet intervention were measured by the $t \times$ time interaction in the random-effects framework. We tested whether the individual differences varied by diet (denoted as $P^2$). This value and the statistical significance of the change with duration of diet change (denoted as $P^3$) are also provided. For each biomarker of interest, the results obtained when all time points were included (baseline, weeks 2, 4, 6 and 8) are shown on the baseline rows, whereas the results obtained based on baseline and week 8 data are shown on the percentage change rows. To determine whether mean concentrations of any biomarkers differed between the 3 diets after adjustment for changes in body weight, we used analysis of covariance models. All $P$ values presented are two-sided. Calculations were performed by using the SAS statistical software system (version 8; SAS Institute, Cary, NC).

**RESULTS**

Women in the 3 diet groups did not differ significantly in age, body weight, or daily intake of calories, macronutrients, or fiber at baseline (Table 1). During the 8 wk of study, there was a small but significant reduction in body weight in all 3 dietary arms: $-1.4$ kg in the CD group ($P = 0.001$), $-2.0$ kg in the SFD group ($P < 0.0001$), and $-2.1$ kg in the VLFD group ($P < 0.0001$). This reduction did not differ significantly between the 3 diet groups. During the study period, intake of fat, carbohydrates, and protein as a percentage of energy and intake of fiber differed significantly between the 3 diet groups (Table 1).

Baseline urinary isoflavone concentrations were low (1.2–4.0 $\mu$mol/d) and not different significantly between subjects in the 3 diet arms. During the intervention, total urinary isoflavone increased significantly to 32.4 (95% CI: 18.4, 56.9) $\mu$mol/d among subjects in the SFD group but remained low among women in the VLFD (1.3 $\mu$mol/d) and the CD (1.0 $\mu$mol/d) groups. In the SFD group, increases were observed for each specific isoflavone: concentrations of genistein, daidzein, and glycitein increased 14–9, and 6-fold, respectively, during the intervention compared with baseline values (data not shown).

Baseline blood concentrations of cholesterol (total, HDL, and LDL cholesterol) and triacylglycerols were not significantly different in the 3 diet groups (Table 2). At the end of 8 wk of
intervention, total cholesterol decreased significantly in all 3 groups, HDL cholesterol decreased significantly only in the VLFD and SFD groups, and LDL cholesterol decreased significantly only in the VLFD group. Triacylglycerol concentrations decreased in the SFD and CD groups but increased in the VLFD group; none of these changes were statistically significant and they did not differ significantly between the 3 diet groups (Table 2). These changes (baseline compared with week 8) and differences between groups were not statistically significant after adjustment for changes in body weight (data not shown).

There were no significant differences in any of the sex-steroid hormones at baseline between the 3 diet groups (Table 3). In association with the intervention, changes in serum estradiol, serum estrone, and the ratio of estradiol to estrone were not statistically significant, and SHBG concentrations increased nonsignificantly in all 3 diet groups. Changes in estrogen and SHBG concentrations also did not differ significantly between the 3 groups. Androgen concentrations increased nonsignificantly in the SFD group and decreased nonsignificantly in the VLFD and CD groups. Although total and free testosterone concentrations decreased significantly in the CD group, these changes in androgen concentrations did not differ significantly between the 3 groups. None of these results were significantly affected by additional adjustment for changes in body weight (data not shown).

Baseline concentrations of IGF-1 (P = 0.048) and IGFBP-3 (P = 0.047) were significantly higher in the SFD compared with the CD and the VLFD groups (Table 4). In association with the intervention, IGF-1 concentrations increased in the 3 dietary arms and was statistically significant in the VLFD group. Concentrations of IGFBP-3 did not change significantly in any of the 3 dietary arms. Baseline insulin and leptin concentrations did not differ significantly among the 3 groups. Insulin concentrations decreased in all 3 groups, but significantly only in the SFD (−32.7%) and the CD (−20.8%). Leptin concentrations decreased significantly in all 3 groups, from 32.7% in the VLFD group to 47.2% in the CD group. Changes in IGF-I, IGFBP-3, insulin, and leptin concentrations did not differ significantly between the 3 groups. These patterns of changes were not significantly altered by further adjustment for changes in body weight (data not shown).

The statistical significance or nonsignificance obtained by using the random-effects model, which used all time points, was not unlike those that used the baseline and week 8 data (see the baseline and percentage change data for P2 and P3 in Tables 2 and 3 and P2 and P3 in Table 4). We observed no significant treatment × time interaction effects.

**DISCUSSION**

The results of this 8-wk intervention study indicate that a VLFD and an SFD produced very small reductions in serum estrogen concentrations in postmenopausal women; these changes did not differ significantly from those observed in women after a CD. These results were unexpected because the fat intake in the VLFD was very low (11.3% of total energy; Table 1), akin to fat intakes in Japan during the 1950s (23). The amount of soy isoflavones added (50 mg isoflavones or 15 g soy protein in the form of tofu) in the SFD was not dissimilar to soy intakes in Japan (7). The fat intake in the CD and SFD groups did not differ significantly from the fat intake in a Step I diet, which has <30% of energy as fat and <10% of energy as saturated fat (24).
Our findings on the hormonal effects of soy food are not unlike the results of a 3-mo soy intervention study in postmenopausal women, which found minimal changes in concentrations of gonadotropin and SHBG (25). In a meta-analysis of 6 soy-intervention studies in postmenopausal women (26–31), we calculated that circulating estrogen concentrations decreased 9.7% in the soy group compared with 5.8% in the control group (4 of 3 studies had a control group). Although a high soy intake was suggested to decrease urinary genotoxic estrogen metabolites in a 3-mo intervention study in postmenopausal women (32), no association was reported in a 6-mo soy-intervention study (30). We did not measure urinary concentrations of estrogen metabolites in this study. Duncan et al (33) proposed that the ability to produce equol after ingesting soy may be an important determinant of hormonal response to soy intake. However, urinary equol concentrations were found in only 3 of the 17 women in the SFD arm, which limited our ability to conduct meaningful analysis separately for equol producers and nonproducers in this study. Whereas soy does not appear to have any major effects on blood estrogen concentrations, at least in short-term settings, these observations do not necessarily contradict epidemiologic observations of an inverse association between soy food and breast cancer risk because longer-term eating habits of soy are likely to have been captured in these studies (6, 7).

The slight reduction in serum estrogen concentrations in the VLFD group of this study differs from our meta-analysis of 13 studies on dietary fat reduction in which serum estradiol concentrations decreased in premenopausal (−7.4%) (based on 9 studies) and postmenopausal (−23.0%) women (based on 4 studies) (11). In the 4 previous intervention studies in postmenopausal women, the range of fat intake was 10% (34) to 24% of energy (35), and weight loss ranged from 0 (36) to 3.5 kg (34) in 1 mo. The intervention period in our study was short but not unlike that of previous studies; 3-5 mo in one study (37) and 3 wk to 2 mo in the other 3 studies (34–36). An important difference between the present study and the previous studies we reviewed (11) was that dietary intake in the intervention and control groups was tightly controlled during the 8 wk of this study. Recent results from 2 longer-term dietary fat reduction studies in premenopausal women (38, 39) suggest that the hormonal effects of a low-fat diet may be weak or nonexistent.

Serum IGF-I concentrations increased significantly in the VLFD group, but this change did not differ significantly from that in the CD group. Circulating IGF-I and IGFBP-3 concentrations have not been consistently associated with intake of dietary fat, carbohydrates, and soy in cross-sectional studies conducted in Western (40–42) and eastern populations (17, 43). Insulin concentrations decreased in all 3 diet groups, significantly in the SFD and the CD groups, but these reductions did not

### TABLE 2

Geometric mean concentrations of blood lipids at baseline and at the completion of the intervention

<table>
<thead>
<tr>
<th>Variable and time of treatment</th>
<th>Control Step I diet (CD)</th>
<th>Soy food Step diet</th>
<th>Very-low-fat diet</th>
<th>( P^2 ) (interaction)</th>
<th>( P^3 ) (time)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n = 20 )</td>
<td>( n = 17 )</td>
<td>( n = 20 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>5.28 4.92, 5.70</td>
<td>5.21 4.82, 5.65</td>
<td>5.47 5.08, 5.88</td>
<td>0.66 0.56</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Week 8</td>
<td>4.90 4.53, 5.28</td>
<td>4.90 4.48, 5.31</td>
<td>4.90 4.53, 5.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change (%)(^f) ( P^3 )</td>
<td>−8.3 ± 2.9</td>
<td>−6.4 ± 3.1</td>
<td>−10.8 ± 2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triacylglycerols (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.40 1.08, 1.81</td>
<td>1.32 0.99, 1.75</td>
<td>1.55 1.21, 2.01</td>
<td>0.72 0.32</td>
<td>0.13</td>
</tr>
<tr>
<td>Week 8</td>
<td>1.31 1.02, 1.70</td>
<td>1.28 0.97, 1.70</td>
<td>1.56 1.21, 2.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change (%)(^f) ( P^3 )</td>
<td>−7.3 ± 8.8</td>
<td>−6.9 ± 9.5</td>
<td>5.1 ± 8.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.23 1.08, 1.40</td>
<td>1.28 1.11, 1.48</td>
<td>1.17 1.03, 1.34</td>
<td>0.69 0.51</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Week 8</td>
<td>1.14 1.01, 1.30</td>
<td>1.04 0.91, 1.18</td>
<td>1.00 0.88, 1.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change (%)(^f) ( P^3 )</td>
<td>−8.9 ± 5.2</td>
<td>−19.1 ± 5.6</td>
<td>−17.3 ± 5.2</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.19 2.77, 3.63</td>
<td>3.13 2.69, 3.65</td>
<td>3.39 2.98, 3.89</td>
<td>0.69 0.28</td>
<td>0.05</td>
</tr>
<tr>
<td>Week 8</td>
<td>2.90 2.56, 3.29</td>
<td>3.13 2.75, 3.57</td>
<td>3.03 2.67, 3.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change (%)(^f) ( P^3 )</td>
<td>−6.7 ± 4.6</td>
<td>0.0 ± 5.0</td>
<td>−11.6 ± 4.6</td>
<td></td>
<td>0.23 0.03</td>
</tr>
</tbody>
</table>

\(^f\) For difference between the 3 diet groups at baseline (ANOVA for continuous variables).

\(^2\) For the test for time × treatment interaction (ANOVA for continuous variables). The \( P \) value on 8 df obtained by using data from baseline and all time points is shown on the baseline rows. The \( P \) value on 2 df obtained by using data from baseline and week 8 is shown on the percentage change rows.

\(^3\) For the main effect of time (ANOVA for continuous variables). The \( P \) value on 4 df obtained by using data from baseline and all time points is shown on the baseline rows. The \( P \) value on 1 df obtained by using data from baseline and week 8 is shown on the percentage change rows.

\(*\) For paired difference in concentrations at baseline compared with week 8 (paired \( t \) test).
TABLE 3
Geometric mean serum hormone concentrations at baseline and at the completion of the intervention

<table>
<thead>
<tr>
<th>Variable and time of treatment</th>
<th>Control Step I diet (n = 20)</th>
<th>Soy food Step I diet (n = 17)</th>
<th>Very-low-fat diet (n = 20)</th>
<th>( P^1 ) (interaction)</th>
<th>( P^2 ) (time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total estradiol (pg/mL)</td>
<td>( \bar{x} ) 15.3 13.4, 17.5</td>
<td>( \bar{x} ) 15.2 13.2, 17.6</td>
<td>( \bar{x} ) 16.4 14.4, 18.8</td>
<td>0.68 0.90 0.39</td>
<td></td>
</tr>
<tr>
<td>Week 8</td>
<td>14.6 13.1, 16.4</td>
<td>14.6 13.0, 16.5</td>
<td>15.9 14.2, 17.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change (%)(^d)</td>
<td>(-3.7 \pm 5.2)</td>
<td>(-4.2 \pm 5.6)</td>
<td>(-3.7 \pm 5.2)</td>
<td>1.00 0.22</td>
<td></td>
</tr>
<tr>
<td>( P^3 )</td>
<td>0.48</td>
<td>0.46</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free estradiol (pg/mL)</td>
<td>( \bar{x} ) 0.37 0.31, 0.43</td>
<td>( \bar{x} ) 0.41 0.35, 0.50</td>
<td>( \bar{x} ) 0.41 0.35, 0.48</td>
<td>0.53 0.86 0.16</td>
<td></td>
</tr>
<tr>
<td>Week 8</td>
<td>0.34 0.30, 0.39</td>
<td>0.39 0.33, 0.45</td>
<td>0.39 0.34, 0.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change (%)(^d)</td>
<td>(-6.6 \pm 5.3)</td>
<td>(-6.8 \pm 5.7)</td>
<td>(-4.5 \pm 5.3)</td>
<td>0.95 0.06</td>
<td></td>
</tr>
<tr>
<td>( P^3 )</td>
<td>0.22</td>
<td>0.24</td>
<td>0.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrone (pg/mL)</td>
<td>( \bar{x} ) 31.3 26.7, 36.8</td>
<td>( \bar{x} ) 32.4 27.2, 38.6</td>
<td>( \bar{x} ) 36.1 30.8, 42.4</td>
<td>0.46 0.44 0.75</td>
<td></td>
</tr>
<tr>
<td>Week 8</td>
<td>30.5 26.2, 35.7</td>
<td>34.2 28.9, 40.4</td>
<td>33.8 28.9, 39.4</td>
<td>0.47 0.70</td>
<td></td>
</tr>
<tr>
<td>Change (%)(^d)</td>
<td>(-1.8 \pm 6.2)</td>
<td>(+4.4 \pm 6.7)</td>
<td>(-6.8 \pm 6.2)</td>
<td>0.47 0.70</td>
<td></td>
</tr>
<tr>
<td>( P^5 )</td>
<td>0.78</td>
<td>0.51</td>
<td>0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol/estrone</td>
<td>( \bar{x} ) 0.50 0.44, 0.55</td>
<td>( \bar{x} ) 0.49 0.43, 0.54</td>
<td>( \bar{x} ) 0.47 0.41, 0.52</td>
<td>0.72 0.65 0.50</td>
<td></td>
</tr>
<tr>
<td>Week 8</td>
<td>0.50 0.45, 0.55</td>
<td>0.44 0.38, 0.50</td>
<td>0.48 0.43, 0.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change (%)(^d)</td>
<td>(-0.08 \pm 2.0)</td>
<td>(-4.7 \pm 2.2)</td>
<td>(+1.3 \pm 2.0)</td>
<td>0.13 0.35</td>
<td></td>
</tr>
<tr>
<td>( P^5 )</td>
<td>0.97</td>
<td>0.06</td>
<td>0.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone (pg/mL)</td>
<td>( \bar{x} ) 21.5 17.1, 27.1</td>
<td>( \bar{x} ) 19.1 14.9, 24.6</td>
<td>( \bar{x} ) 22.6 17.9, 28.5</td>
<td>0.62 0.09 0.36</td>
<td></td>
</tr>
<tr>
<td>Week 8</td>
<td>19.8 15.6, 25.2</td>
<td>20.0 15.4, 25.9</td>
<td>21.7 17.1, 27.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change (%)(^d)</td>
<td>(-7.6 \pm 3.8)</td>
<td>(+4.3 \pm 4.1)</td>
<td>(-4.6 \pm 3.8)</td>
<td>0.10 0.24</td>
<td></td>
</tr>
<tr>
<td>( P^5 )</td>
<td>0.050</td>
<td>0.30</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free testosterone (pg/mL)</td>
<td>( \bar{x} ) 3.0 2.4, 3.8</td>
<td>( \bar{x} ) 3.4 2.6, 4.3</td>
<td>( \bar{x} ) 3.4 2.7, 4.3</td>
<td>0.76 0.34 0.02</td>
<td></td>
</tr>
<tr>
<td>Week 8</td>
<td>2.6 2.1, 3.4</td>
<td>3.3 2.6, 4.3</td>
<td>3.1 2.5, 4.0</td>
<td>0.24 0.01</td>
<td></td>
</tr>
<tr>
<td>Change (%)(^d)</td>
<td>(-12.6 \pm 4.5)</td>
<td>(-1.0 \pm 4.9)</td>
<td>(-7.1 \pm 4.5)</td>
<td>0.24 0.01</td>
<td></td>
</tr>
<tr>
<td>( P^5 )</td>
<td>0.007</td>
<td>0.81</td>
<td>0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Androstenedione (pg/mL)</td>
<td>( \bar{x} ) 504 421, 603</td>
<td>( \bar{x} ) 512 421, 623</td>
<td>( \bar{x} ) 586 490, 702</td>
<td>0.46 0.81 0.47</td>
<td></td>
</tr>
<tr>
<td>Week 8</td>
<td>463 387, 553</td>
<td>526 434, 639</td>
<td>569 476, 680</td>
<td>0.52 0.45</td>
<td></td>
</tr>
<tr>
<td>Change (%)(^d)</td>
<td>(-8.4 \pm 6.4)</td>
<td>(+2.5 \pm 7.0)</td>
<td>(-2.9 \pm 6.5)</td>
<td>0.52 0.45</td>
<td></td>
</tr>
<tr>
<td>( P^5 )</td>
<td>0.20</td>
<td>0.72</td>
<td>0.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>( \bar{x} ) 49 41, 60</td>
<td>( \bar{x} ) 35 28, 43</td>
<td>( \bar{x} ) 44 36, 53</td>
<td>0.08 0.39(^6) 0.01(^6)</td>
<td></td>
</tr>
<tr>
<td>Week 8</td>
<td>53 44, 64</td>
<td>38 32, 47</td>
<td>46 39, 56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change (%)(^d)</td>
<td>(+8.2 \pm 4.6)</td>
<td>(+9.0 \pm 5.1)</td>
<td>(+4.5 \pm 4.6)</td>
<td>0.77(^6) 0.01(^6)</td>
<td></td>
</tr>
<tr>
<td>( P^5 )</td>
<td>0.082</td>
<td>0.079</td>
<td>0.33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) For difference between the 3 diet groups at baseline (ANOVA for continuous variables).
\(^2\) For the test for time \(\times\) treatment interaction (ANOVA for continuous variables). The \(P\) value on 4 df obtained by using data from baseline and all time points is shown on the baseline rows. The \(P\) value on 2 df obtained by using data from baseline and week 8 is shown on the percentage change rows.
\(^3\) For the main effect of time (ANOVA for continuous variables). The \(P\) value on 4 df obtained by using data from baseline and all time points is shown on the baseline rows. The \(P\) value on 1 df obtained by using data baseline and week 8 is shown on the percentage change rows.
\(^4\) All values are \(\bar{x} \pm\) SE. Paired difference in log hormone concentrations at baseline compared with week 8.
\(^5\) For paired difference in concentrations at baseline compared with week 8 (paired \(t\) test).
\(^6\) Baseline sex hormone binding globulin (SHBG) concentrations were included as a covariate in the analysis.

Differ significantly between the diet groups. Significant reductions in insulin concentration in association with soy supplementation were reported in women with type 2 diabetes (44) but not in soy-supplementation studies of nondiabetic women (27, 30). Whereas leptin concentrations decreased significantly in all 3 diet groups, these changes did not differ significantly between the 3 diet groups. Reduction in leptin concentrations remained after adjustment for changes in body weight during the intervention. Most studies published suggest that fasting and refeeding may change blood leptin concentrations, but less is known about the effects of specific nutrients on leptin concentrations (45). No effect of soy on leptin concentrations was reported in a previous soy-intervention study (46). It is of interest that in a large cross-sectional study in the United States, leptin concentrations increased significantly in a stepwise manner with increasing body mass index (47). Mean leptin concentrations were 26.0 ng/mL in women with a body mass index (kg/m^2) between 30 and <35 and were lower (19.8 mg/mL) in women with a body mass index
between 27.5 and <30. Thus, one interpretation of the current results is that even moderate changes in diet (ie, Step 1 control diet) may have beneficial effects, ie, in terms of changes in leptin and insulin concentrations.

Did noncompliance play any role in these findings? We examined adherence based on urinary isoflavone concentrations in the SFD group and serum lipid changes in all 3 arms. Compliance in the SFD group appeared good because urinary isoflavone concentrations, an accepted specific biomarker of soy intake, in the SFD group appeared good because urinary isoflavone concentrations in the SFD group and serum lipid changes in all 3 arms. Compliance in both the VLFD and CD groups was not significantly different from pub-

<table>
<thead>
<tr>
<th>Variable and time of treatment</th>
<th>Control Step I diet (n = 20)</th>
<th>Soy food Step I diet (n = 17)</th>
<th>Very-low-fat diet (n = 20)</th>
<th>P* (interaction)</th>
<th>Pp (time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I (ng/mL)</td>
<td>Baseline 87 75, 101</td>
<td>111 95, 131</td>
<td>86 74, 100</td>
<td>0.048</td>
<td>0.89(^5) 0.002(^5)</td>
</tr>
<tr>
<td>Week 8 94 81, 108</td>
<td>121 104, 141</td>
<td>94 81, 108</td>
<td>0.073</td>
<td>0.16</td>
<td>0.014</td>
</tr>
<tr>
<td>Change (%)(^6)</td>
<td>7.4 ± 4.1</td>
<td>6.3 ± 4.4</td>
<td>10.4 ± 4.1</td>
<td>0.78(^5) 0.002(^5)</td>
<td></td>
</tr>
<tr>
<td>P*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP-3 (μg/mL)</td>
<td>Baseline 4.28 3.82, 4.78</td>
<td>5.25 4.65, 5.93</td>
<td>4.42 3.96, 4.95</td>
<td>0.047</td>
<td>0.92(^8) 0.19(^8)</td>
</tr>
<tr>
<td>Week 8 4.40 3.98, 4.86</td>
<td>4.92 4.41, 5.49</td>
<td>4.40 3.98, 4.86</td>
<td>0.066</td>
<td>0.16</td>
<td>0.92</td>
</tr>
<tr>
<td>Change (%)(^6)</td>
<td>1.8 ± 4.2</td>
<td>−6.4 ± 4.5</td>
<td>−0.4 ± 4.2</td>
<td>0.38(^8) 0.58(^8)</td>
<td></td>
</tr>
<tr>
<td>P*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin (μIU/mL)</td>
<td>Baseline 9.93 7.67, 12.84</td>
<td>11.94 9.04, 15.77</td>
<td>10.06 7.79, 13.01</td>
<td>0.59</td>
<td>0.18</td>
</tr>
<tr>
<td>Week 8 8.66 6.85, 10.96</td>
<td>8.66 6.75, 11.11</td>
<td>8.60 6.85, 10.80</td>
<td>0.032</td>
<td>0.002</td>
<td>0.018</td>
</tr>
<tr>
<td>Change (%)(^6)</td>
<td>−20.8 ± 9.5</td>
<td>−32.7 ± 10.3</td>
<td>−12.9 ± 9.5</td>
<td>0.37</td>
<td>0.0002</td>
</tr>
<tr>
<td>P*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>Baseline 29.3 22.4, 38.3</td>
<td>27.5 20.5, 36.8</td>
<td>24.1 18.4, 31.5</td>
<td>0.61</td>
<td>0.92</td>
</tr>
<tr>
<td>Week 8 18.3 13.2, 25.2</td>
<td>17.2 12.4, 23.7</td>
<td>19.3 13.6, 27.3</td>
<td>0.001</td>
<td>0.002</td>
<td>0.001</td>
</tr>
<tr>
<td>Change (%)(^6)</td>
<td>−47.2 ± 10.1</td>
<td>−39.5 ± 11.0</td>
<td>−32.7 ± 10.1</td>
<td>0.65</td>
<td>0.0001</td>
</tr>
<tr>
<td>P*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
week and to return uneaten foods, and they donated blood specimens every 2 wk for biomarker measurements. Participants were blinded to the diet regimen to which they were randomly assigned. When we asked subjects at the completion of the study to speculate about the diet regimen to which they were assigned, 25% in the CD group, 36% in the SFD group, and 70% in the VLFD group correctly identified their diet. Thus, only the VLFD group identified their diet more accurately than at random.

There were also several limitations in this study. The baseline measurement of blood hormone, lipid, IGF, insulin, and leptin concentrations was based on a single sample collection. Despite our close monitoring of subjects during the 8 wk of dietary intervention, subjects in all 3 dietary arms experienced a small but statistically significant weight loss (1.4–2.1 kg). We have to assume that our assessment of dietary intake at baseline (based on 3-d food records) was an underestimate of the actual intake because all 3 groups showed significant weight loss despite lower reported caloric intakes at baseline than during the intervention period. During the intervention, fat intake in the CD and SFD groups was 26.2% and 24.7% of energy, respectively—lower than the amount (30% of energy as fat) we had planned. On the basis of the daily diet records and uneaten foods that were returned, many of the participants unexpectedly skipped items such as salad dressing, mayonnaise, and margarine, which were included for their addition to foods. Nevertheless, fat intake in the VLFD group (11.3% of energy as fat) was still 15% lower than that in the CD group (26.2% of energy as fat), which suggests that we should have had reasonable power to detect significant changes in lipid between these 2 diet groups. This would not have influenced our findings in relation to estrogen and SHBG because the magnitude of changes did not differ significantly between the 3 dietary arms. The intervention was only 8 wk long and, thus, the longer-term effects of these dietary changes are not known. Finally, although we may have captured the very-low-fat and soy food contents in our VLFD and SFD groups, our intervention diets may not have reflected the traditional Asian diets in terms of sources of fat, protein, and carbohydrates.

In summary, the present study does not provide evidence that short-term ingestion of soy food or a VLFD, resembling intakes in a traditional Asian diet, significantly reduces estrogen concentrations in postmenopausal women. In all 3 diet groups, insulin and leptin concentrations decreased. Results on the effects of a low-fat intervention diet in the Women’s Health Initiative will be extremely informative in elucidating the longer-term effects of dietary fat reduction in postmenopausal women.

We are extremely grateful to all the study participants and the data collection team at the General Clinical Research Center, including Yolanda Stewart, Carla Flores, and others, and in the Department of Preventive Medicine, including Rachel Waasdorp, Jane Yashiki, and Mei-Ying Lai.

AHW contributed to the conception and design. AHW and MCP obtaining funding. CM and AHW collected data. FZS, SH, PM, and SC measured the outcomes of interest. C-CT managed the data. C-CT, DOS, MCP, and AHW conducted the statistical analysis. AHW, FZS, and MCP interpreted the study results. AHW prepared the results. FZS, CM, C-CT, SH, PM, SC, DOS, and MCP reviewed the manuscript. The authors had no conflicts of interest.

REFERENCES

Plasma arginine concentrations are reduced in cancer patients: evidence for arginine deficiency?1–3

Yvonne LJ Vissers, Cornelis HC Dejong, Yvette C Luiking, Kenneth CH Fearon, Maarten F von Meyenfeldt, and Nicolaas EP Deutz

ABSTRACT

Background: The disturbances leading to cancer cachexia remain to be unraveled. Preliminary evidence suggests that arginine availability in cancer is reduced. However, no valid data are available on plasma arginine concentrations in cancer patients.

Objective: We aimed to determine whether there is evidence for disturbed arginine metabolism in cancer.

Design: We measured plasma arginine concentrations postabsorptively in patients with various types of tumors, hypothesizing that arginine concentrations would be lower than those in age- and sex-matched control subjects. Patients with localized tumors with a range of metabolic implications were studied: breast cancer (no weight loss), colonic cancer (sometimes weight loss), and pancreatic cancer (usually weight loss). Plasma amino acid concentrations were measured by HPLC.

Results: Plasma arginine concentrations were lower in patients with cancer (breast cancer: 80 ± 3 compared with 103 ± 9 μmol/L; colonic cancer: 80 ± 3 compared with 96 ± 7 μmol/L; pancreatic cancer: 76 ± 5 compared with 99 ± 7 μmol/L; P < 0.05 versus respective age- and sex-matched control subjects), irrespective of tumor type, weight loss, tumor stage, or body mass index (correlations with P > 0.05).

Conclusions: Malignant tumors associated with various degrees of metabolic derangements are all associated with decreased plasma arginine concentrations, even without weight loss. This suggests that decreased arginine availability is a specific feature of the presence of cancer. These disturbances in arginine metabolism could contribute to the cascade of metabolic events leading to cancer cachexia. Am J Clin Nutr 2005;81:1142–6.

KEY WORDS Cachexia, arginine, tumor, amino acids, malignancy, breast cancer, colonic cancer, pancreatic cancer

INTRODUCTION

Up to one-third of cancer patients die from cachexia rather than from cancer itself, even after surgical removal of the tumor (1). This suggests that cancer-related alterations in metabolism of the host are an important factor in determining mortality. Unfortunately, attempts to reverse cachexia by supplying large amounts of protein or calories have been unsuccessful. Therefore, more detailed insight into the disturbances in host metabolism of cancer patients is necessary.

Evidence is accumulating that the amino acid arginine is of importance in cancer. Both arginine and its product nitric oxide (NO) are important mediators in the defense against tumor cells, because both influence T cell–mediated immunity (2, 3), cytokine induction (4), and macrophage-mediated tumor toxicity (5). Besides, various malignant tumor tissues contain considerable amounts of the enzyme arginase (6–8), which converts arginine to ornithine and urea. It was recently shown that the high arginase activity of tumors is a mechanism of tumor-induced immunosuppression through depletion of arginine concentrations in the microenvironment of the tumor (9). However, it is not known whether this disturbs the arginine metabolism of the host as well. Changes in systemic arginine concentrations of cancer patients could indicate whether the high arginase activity in the proximity of the tumor affects arginine metabolism on the whole-body level as well.

Plasma arginine concentrations have been studied in cancer patients before, but interpretation of these data may not be valid because of incorrect collection of samples and the use of improper control groups. Thus, to determine whether there is evidence for disturbed arginine metabolism in cancer, we measured arginine concentrations in venous blood samples collected postabsorptively from patients with various types of tumors and compared these with values obtained for age- and sex-matched controls collected previously in our laboratory. We aimed to collect blood samples in a way that prevented decomposition of samples and minimized variations in concentrations due to confounding variables. Three tumor types with a range of metabolic implications were studied: breast tumors (no weight loss), colonic tumors (sometimes weight loss), and pancreatic tumors (often weight loss).

SUBJECTS AND METHODS

Patients

Patients with breast cancer (n = 22) or colonic cancer (n = 9) were recruited from the Department of Surgery of the University

1 From the Departments of Surgery, Nutrition, and Toxicology, Research Institute Maastricht, Maastricht University, and University Hospital Maastricht, Maastricht, Netherlands (YLJV, YCL, CHCD, MFvM, and NEPD), and the Royal Infirmary, Edinburgh, United Kingdom (CHCD and KCHF).
2 Supported by grants from the Netherlands Organisation for Health Research and Development (ZonMw, grant numbers 907-00-033 and 920-03-240) and the Niels Stensen Foundation.
3 Address reprint requests to NEP Deutz, Department of Surgery, Maastricht University, PO Box 616, 6200 MD Maastricht, Netherlands. E-mail: nep.deutz@ah.unimaas.nl.
Received November 10, 2004.
Accepted for publication December 14, 2004.

ARGinine DEFICIENCY IN CANcer

**TABLE 1**

Characteristics of the patients and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Breast cancer patients (n = 22)</th>
<th>Colonic cancer patients (n = 9)</th>
<th>Pancreatic cancer patients (n = 21)</th>
<th>Control subjects (pancreas) (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>59 ± 10 (43–74) (^1)</td>
<td>72 ± 10 (55–84)</td>
<td>67 ± 8 (55–82)</td>
<td>68 ± 10 (45–86)</td>
</tr>
<tr>
<td>Sex (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>100</td>
<td>18</td>
<td>33</td>
<td>35</td>
</tr>
<tr>
<td>Male</td>
<td>0</td>
<td>82</td>
<td>67</td>
<td>65</td>
</tr>
<tr>
<td>Tumor stage (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 or 1</td>
<td>27</td>
<td>22</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>T2 or 2</td>
<td>32</td>
<td>0</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>T3 or 3</td>
<td>36</td>
<td>67</td>
<td>14</td>
<td>—</td>
</tr>
<tr>
<td>T4 or 4</td>
<td>0</td>
<td>11</td>
<td>76</td>
<td>—</td>
</tr>
<tr>
<td>Weight loss (kg)</td>
<td>1 ± 2 (0–5)</td>
<td>3 ± 4 (0–12)</td>
<td>13 ± 10 (0–38) (^2)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

\(^1\) x ± SD; range in parentheses (all such values).

\(^2\) Significantly different from age- and sex-matched control subjects, \(P < 0.05\) (Mann-Whitney \(U\) test).

Hospital Maastricht from 2003 to 2004. Patients with pancreatic cancer (n = 21) and contemporary age- and sex-matched control subjects (n = 17) were recruited from the Department of Surgery of the Royal Infirmary of Edinburgh from 1999 to 2000. Exclusion criteria were previous therapy (surgery, chemotherapy, or radiotherapy), metastatic disease, and known metabolic or endocrine disease. The Medical Ethical Committee of the University Hospital Maastricht and the Royal Infirmary of Edinburgh approved the study. Written informed consent was obtained from all patients. Diagnosis of malignancy was confirmed by histologic examination of tumor tissue after surgical removal. Tumor stages were classified according to the guidelines of the American Joint Committee on Cancer. Patient characteristics are summarized in Table 1. Amino acid concentrations of all patient groups were compared with a data set of age- and sex-matched control subjects collected previously by our laboratory (10) and referred to as historical controls.

**Handling of samples**

All blood samples were collected in the morning after the subjects had fasted overnight. Blood was collected from a peripheral vein into precooled heparin-containing tubes and was immediately put on ice. Body weight and length were measured, and patients were asked about any weight loss in the previous 6 mo. Within 0.5 h of sampling, the samples were centrifuged for 10 min at 4000 x g, 4 °C, and the plasma was deproteinized by using sulfosalicylic acid (8 mg per 100 μL plasma). Samples were subsequently put into liquid nitrogen and stored at −80 °C until analyzed. Amino acid concentrations were measured in plasma by using HPLC as described by van Eijk (11) with variation coefficients of <3%.

**Statistical analysis**

Mann-Whitney \(U\) tests were used to test for differences between groups with malignant tumors and their age- and sex-matched controls, hypothesizing that cancer would decrease amino acid concentrations. Correlations between amino acid concentrations and tumor stage were tested by using Kendall’s \(τ\); correlations between amino acid concentrations and weight loss or body mass index (BMI) were tested by using Spearman’s \(ρ\). Significance was defined as \(P < 0.05\).

**RESULTS**

**Patient characteristics**

Of the patients with breast cancer, 4 of 22 had experienced weight loss in the past 6 mo. Four of the 9 patients with colonic cancer had lost weight in the past 6 mo. None of the patients in these 2 groups had lost >10% of their body weight, and all subjects had BMIs (in kg/m²) greater than 21. Of the patients with pancreatic cancer, all but one had lost weight in the past 6 mo. Twelve of 21 had lost >10% of their initial body weight, and 5 patients had BMIs < 19. All age- and sex-matched control groups were weight stable, and BMI averaged 25 ± 1 in each group.

**Amino acid concentrations**

Most plasma amino acid concentrations of pancreatic cancer patients were significantly lower than those of the contemporary and historical controls (Table 2). Values did not differ significantly between the contemporary and historical pancreatic cancer controls for most amino acids. In all patients with malignant disease, plasma arginine concentrations were lower than in age- and sex-matched controls (Figure 1). Branched-chain amino acid concentrations were also lower in all cancer patients. Total amino acid concentrations were lower only in patients with pancreatic cancer (Table 2).

**Correlations**

There was no significant correlation between arginine or total amino acid concentrations and tumor stage, weight loss, or BMI in any patient group.

**DISCUSSION**

The results of the present study show that plasma arginine concentrations are reduced in patients with cancer, even without weight loss being present and irrespective of tumor type, tumor stage, or BMI. Our results suggest that high arginase activity around the tumor affects host arginine metabolism on a whole-body level, indicating a prominent role for altered arginine metabolism in cancer.
Plasma arginine concentrations in various types of cancer have been investigated before. These have been reported to be decreased in lung cancer (12), higher in breast cancer (7, 13), unchanged (13) or higher (14) in gastrointestinal cancer, unchanged in head and neck cancer (13), and unchanged in esophageal cancer (12), as shown in Table 3. However, none of these studies reported that plasma samples were deproteinized before storage, which leaves the enzymatic activity of arginase (which converts arginine to ornithine) intact. As we have shown before, ongoing arginase activity in plasma can decrease arginine concentrations up to 94% (10). This could explain why plasma arginine concentrations were already relatively low in control groups reported in the literature (Table 3). Thus, interpretation of these data may be invalid. To prevent decomposition by enzymatic activity, samples must be deproteinized immediately and stored at −80 °C until analyzed. Moreover, the sampling site in a clinical situation, however, it can be difficult to obtain samples from healthy control groups with age- and sex-matched controls should be at least age- and sex-matched, because we showed that amino acid concentrations vary with the sex of the individual and in-creasing with age (10). Only Naini et al’s study used age-matched controls and observed that plasma arginine concentrations were lower in patients with lung cancer and unchanged in patients with esophageal cancer (12).

In a clinical situation, however, it can be difficult to obtain samples from healthy control groups with age- and sex-matched controls reported in the literature (Table 3). Thus, interpretation of these data may be invalid. To prevent decomposition by enzymatic activity, samples must be deproteinized immediately and stored at −80 °C until analyzed. Moreover, the sampling site in a clinical situation, however, it can be difficult to obtain samples from healthy control groups with age- and sex-matched controls should be at least age- and sex-matched, because we showed that amino acid concentrations vary with the sex of the individual and in-creasing with age (10). Only Naini et al’s study used age-matched controls and observed that plasma arginine concentrations were lower in patients with lung cancer and unchanged in patients with esophageal cancer (12).

**Table 2**

Postabsorptive venous plasma amino acid concentrations

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Breast cancer</th>
<th>Colonic cancer</th>
<th>Pancreatic cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Historical controls</td>
<td>Patients</td>
<td>Historical controls</td>
</tr>
<tr>
<td>Glu</td>
<td>(n = 22)</td>
<td>(n = 22)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>Asn</td>
<td>51 ± 6</td>
<td>63 ± 5</td>
<td>54 ± 6</td>
</tr>
<tr>
<td>Ser</td>
<td>97 ± 9</td>
<td>108 ± 3</td>
<td>90 ± 8</td>
</tr>
<tr>
<td>Gln</td>
<td>654 ± 29</td>
<td>599 ± 14</td>
<td>667 ± 27</td>
</tr>
<tr>
<td>Gly</td>
<td>272 ± 43</td>
<td>276 ± 21</td>
<td>207 ± 32</td>
</tr>
<tr>
<td>Thr</td>
<td>121 ± 8</td>
<td>111 ± 5</td>
<td>112 ± 11</td>
</tr>
<tr>
<td>His</td>
<td>81 ± 4</td>
<td>84 ± 2</td>
<td>76 ± 6</td>
</tr>
<tr>
<td>Cit</td>
<td>35 ± 3</td>
<td>34 ± 2</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>Ala</td>
<td>372 ± 39</td>
<td>351 ± 16</td>
<td>337 ± 41</td>
</tr>
<tr>
<td>Tau</td>
<td>44 ± 3</td>
<td>49 ± 2</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>aAbA</td>
<td>19 ± 2</td>
<td>19 ± 1</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>Tyr</td>
<td>62 ± 4</td>
<td>55 ± 4</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>Val</td>
<td>227 ± 12</td>
<td>181 ± 6^2</td>
<td>233 ± 12</td>
</tr>
<tr>
<td>Met</td>
<td>23 ± 1</td>
<td>20 ± 1</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>Ile</td>
<td>59 ± 5</td>
<td>55 ± 2</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>Phe</td>
<td>58 ± 3</td>
<td>48 ± 1^2</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>Trp</td>
<td>51 ± 2</td>
<td>42 ± 2^2</td>
<td>52 ± 3</td>
</tr>
<tr>
<td>Leu</td>
<td>122 ± 6</td>
<td>99 ± 3^2</td>
<td>128 ± 7</td>
</tr>
<tr>
<td>Lys</td>
<td>162 ± 14</td>
<td>165 ± 6</td>
<td>158 ± 14</td>
</tr>
<tr>
<td>Orn</td>
<td>—</td>
<td>52 ± 2</td>
<td>—</td>
</tr>
<tr>
<td>BCAA</td>
<td>408 ± 212</td>
<td>335 ± 10^2</td>
<td>424 ± 21</td>
</tr>
<tr>
<td>SUMAA</td>
<td>2660 ± 121</td>
<td>2482 ± 48</td>
<td>2555 ± 108</td>
</tr>
</tbody>
</table>

^1 All values are ± SEM. Cit, citrulline; Tau, taurine; aAbA, α-amino butyric acid; Orn, ornithine; BCAA, branched-chain amino acids; SUMAA, total amino acids, excluding Orn.

^2 Significantly different from age- and sex-matched historical controls, P < 0.05 (Mann-Whitney U test).

^3 Significantly different from age- and sex-matched controls, P < 0.05 (Mann-Whitney U test).

**Figure 1.** Mean (±SEM) postabsorptive venous plasma arginine concentrations from patients with various types of malignant tumors and from age- and sex-matched historical control subjects. Breast cancer patients and historical controls, n = 22 per group; colonic cancer patients and historical controls, n = 9 per group; pancreatic cancer patients and historical controls, n = 21 per group. For pancreatic cancer patients, a contemporary group of age- and sex-matched controls was also included (n = 17). *Significantly different from age- and sex-matched controls without malignant disease, P < 0.05 (Mann-Whitney U test).
subjects. Therefore, the use of historical controls of various age groups is an acceptable alternative. Most amino acid concentrations in contemporary controls of the pancreatic cancer patients were not significantly different from the concentrations in historical controls (Table 2). This indicates that there are no major differences in amino acid patterns between the 2 control groups and that the historical controls can be used for comparison with current patient data. Thus, our study is the first to report plasma arginine concentrations in cancer patients with the use of strict methods for collection and storage of samples and with the use of proper control groups.

Evidence for abnormal arginine metabolism in cancer has accumulated. As such, various malignant tissues, such as lung (6), skin (15), prostate (16), colon, and breast tumors (7, 8), contain high amounts of arginase. It was shown recently that arginase activity in tumors induces arginine deficiency in the microenvironment of the tumor, which enables tumors to escape the immune response (9). However, it is not known whether this has systemic effects on arginine metabolism. The low arginine concentrations in cancer patients suggest that the presence of a tumor indeed affects arginine metabolism on a whole-body level. Whether this is due to increased arginine uptake by the tumor or the secretion of arginase into the circulation remains to be determined.

Apart from arginase, arginine can be converted by NO synthase (NOS) to citrulline and NO. NO is the effector molecule to which many of the functions of arginine are ascribed. For example, NO is the effector molecule of the tumor-cytotoxic mechanism of macrophages (5). Therefore, arginine deficiency could decrease NO production and have adverse effects in cancer patients. On basis of the Michaelis constant ($K_m$) for NOS (2–20 $\mu$mol/L), the observed decrease in arginine concentrations in cancer patients should not limit NO production. However, the existence of caveolar complexes between NOS and membrane transporters of arginine (17) suggests that arginine transporters may be more important than the $K_m$ of NOS for regulation of NO production. Because these arginine transporters have $K_m$ values in the range of physiologic arginine concentrations (18), decreases in arginine concentrations such as observed in the present study may indeed limit NO production.

Disturbances in arginine and NO metabolism in the presence of cancer can be unfavorable, especially when cancer patients undergo treatments that result in tissue damage, such as surgical removal of the tumor or radio- or chemotherapy. Because arginine has been shown to stimulate the immune system (19) and anabolism (3, 20), abnormal arginine metabolism can disturb repair responses such as the acute-phase protein response. We recently observed that low arginine concentrations in tumor-bearing mice prohibit the postoperative increase in arginine and NO production, concomitant with a suppressed acute-phase response (YLJ Vissers, unpublished observation, 2004). This finding indicates that metabolic disturbances become even more pronounced when metabolism is challenged to respond to stress.

There was no relation between arginine or total amino acid concentrations and tumor stage or previous weight loss. Strikingly, plasma arginine concentrations were decreased in all tumor types studied, including breast and colonic cancer, which have always been assumed to cause little metabolic derangements. Moreover, the decreased arginine concentrations were specific, because total amino acid concentrations were maintained. Furthermore, previous studies from our group investigating amino acid metabolism in patients with chronic obstructive pulmonary disease did not show changes in plasma arginine concentrations compared with healthy age- and sex-matched controls (21, 22). Moreover, there was no relation between plasma arginine concentrations and BMI, weight loss, or fat-free mass (21), which indicates that the observed arginine depletion in the presence of a tumor is probably specific for cancer. In the pancreatic cancer patients, in whom weight loss was present, all amino acid concentrations were decreased. This suggests that arginine concentrations change early in the course of tumor development and could indicate that this contributes to the initiation of the cascade of metabolic disturbances leading to cachexia.

Other factors playing a role may well be branched-chain amino acid or essential amino acid metabolism. Obviously, further studies are necessary to elucidate whether derangements in arginine metabolism are causally related to the phenotypic characteristics of cachexia.

Over the past decade, it has become clear that the host’s metabolism in cancer is different from that in simple starvation or

### TABLE 3

Data from the literature on plasma arginine concentrations in cancer

<table>
<thead>
<tr>
<th>Authors and reference</th>
<th>Tumor site</th>
<th>Weight loss</th>
<th>Cancer patients</th>
<th>Control subjects</th>
<th>Storage at $-80^\circ$C</th>
<th>One pool</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass et al (14)</td>
<td>Gastrointestinal tract</td>
<td>Yes</td>
<td>70 ± 4$^2$</td>
<td>48 ± 4</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Naini et al (12)</td>
<td>Esophagus</td>
<td>?</td>
<td>79 ± 7</td>
<td>84 ± 3</td>
<td>No</td>
<td>?</td>
</tr>
<tr>
<td>Park et al (7)</td>
<td>Breast</td>
<td>?</td>
<td>167 ± 17</td>
<td>111 ± 10</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Kubota et al (13)</td>
<td>Head-neck</td>
<td>Yes</td>
<td>77 ± 9 (F)</td>
<td>83 ± 6 (F)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Brodie et al (8)</td>
<td>Breast</td>
<td>No</td>
<td>109 ± 7</td>
<td>77 ± 7 (M)</td>
<td>83 ± 8 (M)</td>
<td></td>
</tr>
</tbody>
</table>

$^1$ None of the samples were deproteinized. None of the control groups were age- and sex-matched. None of the authors reported whether samples were collected at one time point. Question mark indicates “not mentioned.”

$^2$ $\bar{x}$ ± SEM (all such values).
fasting, and that mere supplementation of calories cannot reverse cachexia. This knowledge resulted in studies investigating the metabolic changes accompanying weight loss in cancer. Still, the mechanism of cancer cachexia remains to be unraveled, which might be because of the complexity of the metabolic abnormalities that are present when symptoms such as weight loss or anorexia become obvious. Therefore, studying early changes in cancer may provide insight into the onset of the events that ultimately lead to cachexia. In this respect, it is clear from the present data that metabolic changes were already apparent in patients with breast or colonic cancer without weight loss being present. Our observation supports the idea of cancer inducing disturbances before anorexia and loss of muscle and fat become obvious. In fact, data from noncachectic patients may give better clues to the factors involved in initiating cachexia rather than accompanying it. In this respect, disturbances in arginine metabolism may play a role.

In conclusion, plasma arginine concentrations are decreased in cancer patients both with and without weight loss, which indicates that arginine metabolism is disturbed in the presence of a malignant tumor. Further study of in vivo production rates of arginine and NO in cancer patients is indicated.

We thank DJH Haagen, P de Boer, and HMH van Eijk for expert HPLC measurements and JM Maessen and MMH Hendrikx for assistance with collection of blood samples taken in Maastricht.

YLJV and CHCD collected the patient’s blood samples; YLJV and NEPD wrote the manuscript; and all authors contributed to study design, data interpretation, and final setup of the manuscript. None of the authors had advisory board affiliations or financial interests in organizations sponsoring the research.

REFERENCES

Dairy, calcium, and vitamin D intakes and prostate cancer risk in the National Health and Nutrition Examination Epidemiologic Follow-up Study cohort1–3

Marilyn Tseng, Rosalind A Breslow, Barry I Graubard, and Regina G Ziegler

ABSTRACT

Background: Dairy intake may increase prostate cancer risk, but whether this is due to calcium’s suppression of circulating vitamin D remains unclear. Findings on calcium and vitamin D intake and prostate cancer are inconsistent.

Objective: We examined the association of dairy, calcium, and vitamin D intake with prostate cancer.

Design: In a prospective study of 3612 men followed from 1982–1984 to 1992 for the first National Health and Nutrition Examination Epidemiologic Follow-up Study, 131 prostate cancer cases were identified. Dietary intake was estimated from questionnaires completed in 1982–1984. Relative risk (RR) and 95% CIs were estimated by using Cox proportional hazards models adjusted for age, race, and other covariates.

Results: Compared with men in the lowest tertile for dairy food intake, men in the highest tertile had a relative risk (RR) of 2.2 (95% CI: 1.2, 3.9; trend P = 0.05). Low-fat milk was associated with increased risk (RR = 1.5; 95% CI: 1.1, 2.2; third compared with first tertile; trend P = 0.02), but whole milk was not (RR = 0.8; 95% CI: 0.5, 1.3; third compared with first tertile; trend P = 0.35). Dietary calcium was also strongly associated with increased risk (RR = 2.2; 95% CI: 1.4, 3.5; third compared with first tertile; trend P = 0.001). After adjustment for calcium intake, neither vitamin D nor phosphorus was clearly associated with risk.

Conclusions: Dairy consumption may increase prostate cancer risk through a calcium-related pathway. Calcium and low-fat milk have been promoted to reduce risk of osteoporosis and colon cancer. Therefore, the mechanisms by which dairy and calcium might increase prostate cancer risk should be clarified and confirmed. Am J Clin Nutr 2005;81:1147–54.

KEY WORDS Dairy, diet, calcium, vitamin D, prostatic neoplasms

INTRODUCTION

Both ecologic (1) and epidemiologic studies (2) have fairly consistently found an increase in prostate cancer risk with intake of dairy foods. A strong ecologic correlation between milk intake and prostate cancer mortality was noted as early as 1975 (1), and in a more recent ecologic analysis, the correlation was stronger for milk and prostate cancer mortality than for any other dietary factor, including red meat (3). Among epidemiologic studies, 7 of 10 prospective studies found a positive association between dairy intake and prostate cancer risk (2, 4). Studies that examined individual types of dairy products show more consistent findings for milk (2), probably because milk is the most commonly consumed form of dairy. Although initial explanations for the observed dairy effect related to the fat content in dairy foods, the hypothesis that 1,25-dihydroxyvitamin D (1,25-D) might protect against prostate cancer (5) suggests another possible mechanism: that at sufficiently high amounts, dietary calcium suppresses production of 1,25-D, thereby increasing risk of prostate cancer (6).

The observation that dairy may increase risk of prostate cancer is troubling, given current dietary recommendations for calcium intake (7), aggressive promoting of dairy as a source of calcium (8), and the possibility that calcium intake may protect against colon cancer (9). The objective of this analysis was to examine the associations of dairy food, calcium, and vitamin D intake with prostate cancer risk, to determine whether previous findings can be confirmed, and to assess the extent to which associations observed for dairy might be due to their calcium content, possibly through a vitamin D-related pathway.

SUBJECTS AND METHODS

Study population

The study sample included male participants in the first National Health and Nutrition Examination Survey (NHANES I) Epidemiologic Follow-up Study (NHEFS). NHANES I, conducted between 1971 and 1975, used a multistage sampling design to obtain a national probability sample of the noninstitutionalized civilian population of the United States, excluding Alaska, Hawaii, and Native American reservation lands (10, 11). The elderly and persons residing in poverty areas were oversampled. Of the persons selected ≈70% were both interviewed and medically examined in NHANES I.

1 From the Division of Population Science, Fox Chase Cancer Center, Philadelphia, PA (MT); the National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD (RAB); and the Epidemiology and Biostatistics Program, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD (BIG and RGZ).

2 Supported by US Public Health Service grant CA-06927 from the National Institutes of Health, grant IRG-92-027 from the American Cancer Society, grant DAMD17-01-0057 from the Department of Defense, and an appropriation from the Commonwealth of Pennsylvania.

3 Address reprint requests to M Tseng, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111. E-mail: m_tseng@fccc.edu. Received September 9, 2004. Accepted for publication December 23, 2004.
NHEFS was a longitudinal study of the 14,407 NHANES I participants between the ages of 25 and 74 y at the time of the initial survey (12–15). Participants were followed for health and vital status through 1992. At interviews conducted in 1982–1984, 1986, 1987, and 1992, participants or their proxies were interviewed. Also, health records were obtained for overnight stays in a health care facility occurring after the baseline examination. Through the National Death Index and other tracing mechanisms, death certificates were obtained for deaths during the follow-up period. Health records were obtained for >70% of overnight stays reported by subjects, and death certificates were obtained for 99% of deaths occurring between 1971–1975 and the 1992 follow-up (15). The procedures followed for NHEFS were in accordance with the ethical standards of the National Center for Health Statistics, which conducted the survey, and approval for the survey was obtained from the center’s Ethical Review Board.

Because the 1982–1984 interview included more detailed questions on dietary habits and intake than the interview conducted in 1971–1975, the 1982–1984 interview served as the baseline for these analyses. Of the 14,407 NHEFS participants, 5811 were men. Of these, 1202 had died before the 1982–1984 interview, 351 could not be traced, and 333 were traced but not interviewed in 1982–1984. Subjects were also excluded if they had a diagnosis of prostate cancer at or before the 1982–1984 interview (n = 79), did not complete the diet questionnaire (n = 57), or reported an energy intake of <500 or >4400 kcal/d (n = 10), which left 3779 men available for analysis.

Identification of prostate cancer cases

Cases of invasive prostate cancer were identified, following a procedure described by Breslow et al (16). Briefly, potential cases were all men with an International Classification of Diseases, Ninth Revision, Clinical Modification code of 185 (invasive prostate cancer), 233.4 (prostate carcinoma in situ), v10.46 (personal history of malignant prostate neoplasm), or 60.3–60.5 (prostatectomy surgical procedures) recorded in at least one of the following ways: 1) a first diagnosis of prostate cancer reported at any of the follow-up interviews conducted in 1986, 1987, or 1992; 2) at least 1 hospital stay during the follow-up period with a discharge diagnosis coded as any of the above-mentioned codes; or 3) a death certificate with underlying or nonunderlying cause of death coded as any of the above-mentioned codes. Archived records of interviews and overnight stays in a health care facility were then reviewed. None of the prostate cancer cases identified were in situ cases. “Definite” case status was assigned if a diagnosis of prostate cancer could be confirmed from histopathology reports or medical records, whereas determinations that were based only on interview or death certificate data were assigned “probable” case status. Of 136 cases diagnosed during follow-up of the 3779 men between 1982–1984 and 1992, 89 were considered definite cases, and 47 were considered probable cases.

Data collection

Information on dietary intake was obtained from a 105-item food-frequency questionnaire administered in the 1982–1984 interview. The questionnaire included 7 dairy items: whole or evaporated milk; low-fat, skim, dry, or buttermilk; cheese or cheese dishes; yogurt; cream or sour cream; cottage cheese; and ice cream. Intake of specific nutrients such as energy, calcium, and phosphorus was estimated by multiplying frequency of intake of each food by the nutrient content for the food’s portion size. Because the 1982–1984 NHEFS dietary interview collected only frequency information, information on nutrient content and portion size for each food item was based on sex- and age-specific 24-h recall data from the second National Health and Nutrition Examination Survey (NHANES II), a separate national survey conducted in 1976–1980. A detailed description of the method used to assign nutrient content and portion size to each food item in the NHEFS dietary questionnaire by using NHANES II data has been published (17).

We used the same procedures to assign sex- and age-specific vitamin D content per portion size to food items. International Units (IU) of vitamin D per 100 g food were based on the current US Department of Agriculture (USDA) nutrient database (18), supplemented with other published values (19). USDA values for vitamin D are based primarily on published data from 1991 (20), with some values updated for ready-to-eat cereals (18). Food intake for our study sample was assessed in 1982–1984, but, with the exception of ready-to-eat cereals, vitamin D values are unlikely to have changed substantially for the principal sources between 1982–1984 and 1991: vitamin D in seafood occurs naturally, and the recommendation that fortified milk contains 400 IU/qt has been in place since 1957 (21).

To estimate the amount of vitamin D in foods with vitamin D–containing ingredients such as cheese dishes or milk-containing baked goods, we used recipes available from the USDA Survey Nutrient Database for the 1994–1996 Continuing Survey of Food Intakes by Individuals (22) and other recipe sources (23, 24). Because dairy products used as ingredients in commercial items may not all be vitamin D–fortified (The National Dairy Council, personal communication, 2002), we assumed that milks and cheeses in commercially made items were not fortified, and that vitamin D–fortified milks and cheeses were used only in mixed dishes specified as homemade, from a home recipe, or from a mix. Among 56 milk- or cheese-containing commercial food items for which recipes were obtained, the average difference between the commercial items and their homemade counterparts was 10 IU vitamin D/100 g food.

Participants were also asked about their current use in 1982–84 of multivitamins and of any other vitamins, minerals, or nutritional supplements. Supplements were identified as calcium supplements if the name included calcium, bone meal, oyster shell, or dolomite. Regular use of the antacid Tums was also considered use of a calcium supplement. Other information available from the 1982–84 interview included race, current place of residence, longest held occupation, current family income, first-degree family history of prostate cancer, current weight, current alcohol intake, current smoking behavior, current sun exposure, and current level of physical activity. Information on height and level of education was available from the 1971–1975 interview.

Data analysis

Follow-up time was calculated by subtracting the 1982–1984 interview date from date of last interview for noncases or from date of prostate cancer diagnosis for cases. For 4 cases identified from death certificate data only, the 1982–1984 interview date was subtracted from date of death rather than from date of diagnosis.
We used Cox proportional hazards models adjusting for age (continuous years), race (white, black, or other race), and energy intake (tertiles) to estimate relative risk (RR) of prostate cancer for dairy foods and nutrients. Intake of dairy was calculated as the total intake of all 7 dairy food items in the questionnaire. Nutrient values, estimated from dietary sources only, were log-transformed as necessary and energy-adjusted by using the residual method (25). RRs were estimated for tertiles of intake relative to the lowest tertile, but, for infrequently consumed items such as yogurt and cream, estimates were for consumption compared with nonconsumption.

Other variables, including US region (Northeast, Midwest, South, West), residence (urban, rural, or suburban), education (<high school, high school completion, >high school), first-degree family history of prostate cancer, current body mass index, recreational physical activity (little or none, moderate, much), usual level of daily activity (inactive, moderately active, very active), recreational (little, occasional, frequent) and occupational sun exposure, multivitamin use, smoking status (never, former, current), and past and current alcohol consumption (none, little, moderate, heavy), were evaluated as confounders on the basis of their associations with predictor and response variables and by comparing unadjusted and adjusted estimates from regression analyses. Final multivariate models included 3612 men with complete covariate data and adjusted for age; race; energy intake; US region; rural, urban, or suburban residence; education; recreational sun exposure; recreational and usual level of physical activity; smoking status; and current alcohol intake.

$P$ values for trend were obtained for dairy food and nutrient intake by including an ordinal variable that included the median values for each category in the multivariate model controlling for the covariates listed in the preceding paragraph. To examine interactions between variables, we ran proportional hazards models with individuals cross-classified according to the variables of interest, which we dichotomized by grouping together tertiles with similar RR estimates (calcium tertile 3 compared with tertiles 1 + 2; vitamin D tertiles 2 + 3 compared with tertile 1). Although a post hoc decision, dichotomizing the variables in this way assumed that individuals in exposure categories similarly related to prostate cancer risk would show similar effects in relation to a potentially interacting variable and served to limit the number of categories to be compared.

Because of the possibility of inaccurate statistical adjustment in the tertile analysis because of high correlation between calcium and vitamin D intake, we also modeled energy-adjusted calcium and vitamin D as continuous predictors of prostate cancer. Although vitamin D was found to have a linear relation, we used a four-knot restricted cubic regression spline to model a nonlinear relation of calcium intake with risk (26).

Final models were run by using SUDAAN (27) to account for the stratification and cluster sampling of the NHANES I sample design. Unweighted analyses were conducted, but to account for the sample weighting in NHANES I we included the following design variables (variables that determine the sample weighting) (28) as covariates in the analyses: age (<65 compared with ≥65 y), poverty census enumeration district (residence compared with nonresidence), and family income (<$3000, $3000–$6999, $7000–$9999, $10 000–$14 999, and ≥$15 000), although estimates were similar in models without design variables.

### RESULTS

Descriptive characteristics of the study sample with complete covariate data are shown in Table 1. Mean age of the men was 57.8 y, 11% were African American, and their usual residence was roughly equally distributed among the 4 regions of the United States. The men consumed dairy foods almost twice a day on average. The most commonly consumed dairy items were low-fat and whole milk, cheese, and ice cream, whereas cottage cheese, cream, and yogurt were generally eaten less than once a week.

Over a mean follow-up of 7.7 y (range: <1–10.7 y), 131 prostate cancer cases were identified in the cohort of 3612 men. In Cox proportional hazards models (Table 2), dairy food intake (third compared with first tertile RR = 2.2; 95% CI: 1.2, 3.9; trend $P = 0.05$) was strongly associated with prostate cancer risk. When each dairy food was examined individually, the increase in risk was observed for total milk intake (third compared with first tertile RR = 1.8; 95% CI: 1.1, 2.9; trend $P = 0.03$) but for low-fat milk (third compared with first tertile RR = 1.5; 95% CI: 1.1, 2.2; trend $P = 0.02$) in particular. No elevation was observed for any other dairy food item. Because of the modest inverse correlation between low-fat milk and whole milk consumption ($r = -0.20$), we ran models that included both variables to account for possible confounding but saw no meaningful change in estimates.

Dietary calcium was also strongly associated with risk (third compared with first tertile RR = 2.2; 95% CI: 1.4, 3.5; trend $P = 0.001$) (Table 3). In addition, when we looked at calcium from different food sources, only calcium from low-fat milk was

### TABLE 1

Descriptive characteristics and intake of selected foods and nutrients for 3612 adult male participants in the National Health Examination Follow-up Study at baseline, 1982–1984

<table>
<thead>
<tr>
<th>Food intake (servings/wk)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy foods</td>
<td>12.9 ± 9.6</td>
</tr>
<tr>
<td>Total milk (low-fat + whole)</td>
<td>7.4 ± 7.7</td>
</tr>
<tr>
<td>Low-fat milk</td>
<td>3.8 ± 6.1</td>
</tr>
<tr>
<td>Whole milk</td>
<td>3.6 ± 6.1</td>
</tr>
<tr>
<td>Cheese</td>
<td>2.3 ± 2.6</td>
</tr>
<tr>
<td>Ice cream</td>
<td>1.8 ± 2.3</td>
</tr>
<tr>
<td>Cottage cheese</td>
<td>0.7 ± 1.5</td>
</tr>
<tr>
<td>Cream</td>
<td>0.5 ± 1.9</td>
</tr>
<tr>
<td>Yogurt</td>
<td>0.2 ± 1.1</td>
</tr>
</tbody>
</table>

### TABLE 2

Relative risk estimates for total milk intake (third compared with first tertile RR = 2.2; 95% CI: 1.2, 3.9; trend $P = 0.05$)

<table>
<thead>
<tr>
<th>Food intake (servings/wk)</th>
<th>Relative Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy foods</td>
<td>1.8 ± 2.9</td>
</tr>
<tr>
<td>Total milk (low-fat + whole)</td>
<td>1.5 ± 2.2</td>
</tr>
<tr>
<td>Low-fat milk</td>
<td>1.2 ± 1.8</td>
</tr>
<tr>
<td>Whole milk</td>
<td>1.0 ± 1.7</td>
</tr>
<tr>
<td>Cheese</td>
<td>0.9 ± 1.6</td>
</tr>
<tr>
<td>Ice cream</td>
<td>1.1 ± 2.1</td>
</tr>
<tr>
<td>Cottage cheese</td>
<td>0.7 ± 1.4</td>
</tr>
<tr>
<td>Cream</td>
<td>0.5 ± 1.4</td>
</tr>
<tr>
<td>Yogurt</td>
<td>0.3 ± 0.9</td>
</tr>
</tbody>
</table>

### TABLE 3

Relative risk estimates for calcium intake (third compared with first tertile RR = 2.2; 95% CI: 1.4, 3.5; trend $P = 0.001$)

<table>
<thead>
<tr>
<th>Calcium (mg/d)</th>
<th>Relative Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-fat milk</td>
<td>2.1 ± 1.9</td>
</tr>
<tr>
<td>Whole milk</td>
<td>1.7 ± 1.6</td>
</tr>
<tr>
<td>Cheese</td>
<td>1.3 ± 1.2</td>
</tr>
<tr>
<td>Ice cream</td>
<td>1.1 ± 1.5</td>
</tr>
<tr>
<td>Cottage cheese</td>
<td>0.7 ± 1.3</td>
</tr>
<tr>
<td>Cream</td>
<td>0.5 ± 1.1</td>
</tr>
<tr>
<td>Yogurt</td>
<td>0.3 ± 0.8</td>
</tr>
</tbody>
</table>
clearly associated with risk (third compared with first tertile RR = 1.7; 95% CI: 1.1, 2.6; trend P = 0.02), although the association was not as strong as that for total calcium. Calcium from all other dietary sources, including calcium from whole milk, from all other dairy besides milk, and from nondairy sources, was not positively associated with risk (Table 3). We saw no elevation in risk for the 151 men (4%) who reported use of calcium supplements (RR = 0.9; 95% CI: 0.4, 1.5) or for the 846 men (23%) who reported use of multivitamins (RR = 0.9; 95% CI: 0.6, 1.5). Risk was also not especially elevated among 312 men in the highest tertile of calcium intake who were also users of multivitamins or calcium supplements relative to 1067 nonusers in the lowest tertile of calcium intake (RR = 1.9; 95% CI: 0.9, 3.7).

Phosphorus was not associated with risk of prostate cancer when calcium was also considered (third compared with first tertile RR = 0.9; 95% CI: 0.5, 1.6; trend P = 0.77), nor did we see evidence for any interaction between phosphorus and calcium intake. In contrast, with adjustment for calcium intake, vitamin D was inversely, although not significantly, associated with prostate cancer risk (third compared with first tertile RR = 0.6; 95% CI: 0.3, 1.2; trend P = 0.16; Table 3). Risk did not decrease, however, with intake of the principal food sources of vitamin D, namely low-fat or whole milk, fish, or shellfish, even

**TABLE 2**

Adjusted relative risk (RR) estimates and 95% CIs for prostate cancer by tertile of dairy food intake for 3612 adult male participants in the National Health Examination Follow-up Study followed from 1982–1984 to 1992

<table>
<thead>
<tr>
<th>Dairy</th>
<th>Median intake</th>
<th>Cases</th>
<th>Person-years</th>
<th>Minimal model RR (95% CI)</th>
<th>Full model RR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy servings/wk</td>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tertile 1</td>
<td>5</td>
<td>32</td>
<td>9402</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Tertile 2</td>
<td>11</td>
<td>38</td>
<td>9642</td>
<td>1.2 (0.7, 2.0)</td>
<td>1.1 (0.7, 1.9)</td>
</tr>
<tr>
<td>Tertile 3</td>
<td>21</td>
<td>61</td>
<td>8770</td>
<td>2.3 (1.3, 4.2)</td>
<td>2.2 (1.2, 3.9)</td>
</tr>
<tr>
<td><em>P</em> for trend</td>
<td></td>
<td></td>
<td></td>
<td>0.003</td>
<td>0.05</td>
</tr>
<tr>
<td>Total milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tertile 1</td>
<td>0.5</td>
<td>34</td>
<td>9894</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Tertile 2</td>
<td>7</td>
<td>47</td>
<td>10415</td>
<td>1.2 (0.7, 2.0)</td>
<td>1.1 (0.7, 1.8)</td>
</tr>
<tr>
<td>Tertile 3</td>
<td>14</td>
<td>50</td>
<td>7505</td>
<td>1.9 (1.2, 3.2)</td>
<td>1.8 (1.1, 2.9)</td>
</tr>
<tr>
<td><em>P</em> for trend</td>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>Low-fat milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tertile 1</td>
<td>0</td>
<td>58</td>
<td>13220</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Tertile 2</td>
<td>1</td>
<td>15</td>
<td>5349</td>
<td>0.9 (0.5, 1.6)</td>
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1 Adjusted for age, race, energy intake, and design variables.
2 Additionally adjusted for US region; rural, urban, or suburban residence; education; recreational sun exposure; recreational and usual level of physical activity; smoking status; and current alcohol intake.
3 Obtained by including in the model a variable representing the median value for each tertile.
with adjustment for calcium intake. Because of concern about inaccurate risk estimates because of the substantial correlation (Pearson $r = 0.79$) between calcium and vitamin D intake, we computed additional models that included both as continuous variables and used a four-knot spline to model a nonlinear relation of calcium intake with prostate cancer risk. In these analyses, vitamin D was no longer associated with risk, but the strong positive association for calcium persisted (results not shown).

Current use of cod liver oil was also not associated with prostate cancer risk ($RR = 1.0; 95\% CI: 0.2, 4.5$), but only a small number of men ($n = 50$) reported its use. We found no evidence of effect modification when we examined relative risks for individuals cross-classified according to both calcium and vitamin D intake ($P$ for interaction $= 0.59$).

In models for dairy foods that were additionally adjusted for calcium intake, associations for overall dairy (third compared with first tertile $RR = 1.4; 95\% CI: 0.6, 3.4$; trend $P = 0.35$), total milk (third compared with first tertile $RR = 0.9; 95\% CI: 0.4, 1.9$; trend $P = 0.78$), and low-fat milk (third compared with first tertile $RR = 1.1; 95\% CI: 0.7, 1.7$; trend $P = 0.79$) were attenuated, whereas RR estimates and the trend $P$ value for calcium were not meaningfully changed (data not shown).

Because low-fat milk consumption was associated with higher socioeconomic status, we explored the possibility that our findings for low-fat milk might be due to detection bias by controlling for potential surrogates of screening awareness or access, namely, level of education; poverty; and urban, rural, or suburban residence. Additional adjustment for these factors did not meaningfully change

### TABLE 3

<table>
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<tr>
<th>Median intake$^1$</th>
<th>Cases</th>
<th>Person-years</th>
<th>Minimal model RR (95% CI)$^2$</th>
<th>Full model RR (95% CI)$^3$</th>
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1 mg/d for calcium and phosphorus; IU/d for vitamin D.
2 Adjusted for age, race, energy intake, and design variables.
3 Additionally adjusted for US region; rural, urban, or suburban residence; education; recreational sun exposure; recreational and usual level of physical activity; smoking status; and current alcohol intake.
4 Obtained by including in the model a variable representing the median value for each tertile.
5 Includes cheese or cheese dishes, yogurt, cream or sour cream, cottage cheese, and ice cream.
the elevated risks observed for dairy, calcium, low-fat milk, or calcium from low-fat milk. Because of more widespread use of prostate-specific antigen (PSA) testing for prostate cancer screening after 1991, cases diagnosed from 1991 on probably included more early, slow-growing tumors, whereas cases diagnosed before 1991 were more aggressive. When we conducted analyses that included only 107 cases identified before 1991, the associations for low-fat milk (third compared with first tertile RR = 1.4; 95% CI: 1.0, 2.1; trend \( P = 0.06 \)), calcium (third compared with first tertile RR = 2.0; 95% CI: 1.2, 3.4; trend \( P = 0.007 \)), and calcium from low-fat milk (third compared with first tertile RR = 1.6; 95% CI: 1.0, 2.7; trend \( P = 0.05 \)) were not meaningfully altered. Risk estimates were also not materially different when we limited cases more conservatively to the 46 identified before approval by the US Food and Drug Administration of PSA testing in 1986, excluded 14 prostate cancer cases diagnosed within a year of the dietary interview, reclassified 47 probable cases as noncases, or used age rather than time on study as the time scale (29).

Variables reflecting sun exposure that may also determine circulating vitamin D concentrations, including recreational and occupational sun exposure, reaction of the skin to sun exposure, ability to tan, and current residence in the southern region of the United States, were not associated with prostate cancer risk (results not shown). In a separate analysis (30), we saw evidence for an inverse association for intake of a southern pattern of food intake, characterized by such foods as cornbread, grits, sweet potatoes, and okra, possibly a marker of substantial cumulative sunlight exposure through longtime residence in the South (31). However, the southern dietary pattern did not significantly modify the effects of dairy, calcium, or vitamin D in these data. In race-specific analyses, associations of prostate cancer risk with dairy, calcium, and vitamin D intake were also similar.

DISCUSSION

Our findings are consistent with most studies that observed an elevated risk of prostate cancer with greater dairy or milk intake (2) and with several (4, 32–35) but not all (36–42) studies that observed an elevated risk with greater calcium intake. Our risk estimates for dairy and calcium are higher than some previously reported estimates (4, 32, 33) but comparable to those from the Health Professionals Follow-Up Study (34) and from a case-control study in King County, WA (35). In the Health Professionals cohort (34), RR estimates for advanced prostate cancer were 1.6 for \( > 2 \) compared with 0 glasses milk/d, and 1.6 for \( \geq 1000 \) compared with <600 mg Ca from food/d. In the King County, WA, study (35), odds ratio estimates for regional or distant disease were 2.1 for \( > 2 \) compared with \( < 2 \) glasses milk/wk, and 1.6 for \( \geq 838 \) compared with <564 mg Ca from food/d. Effect estimates for calcium from food were more pronounced than for supplemental calcium in 2 (4, 35) of 3 (4, 34, 35) studies.

Notably, several previous studies that included larger proportions of cases diagnosed after the widespread adoption of PSA for screening saw stronger associations with calcium for more advanced disease than for the early, preclinical disease often detected by PSA screening (4, 34, 35). A distinct advantage of the current study is that most of the cases were diagnosed before more widespread PSA screening began in 1991 (43). Cases were, thus, less likely to be diagnosed incidentally and more likely to be advanced and clinically apparent, which allowed for a clearer examination of dairy and calcium intake in relation to clinically relevant disease.

Dairy foods may increase prostate cancer risk by raising circulating concentrations of insulin-like growth factor I (44, 45), but such a mechanism would not explain why we observed an association for low-fat milk only. Alternatively, calcium in dairy may increase risk by suppressing concentrations of circulating 1,25-D (6). Possibly, this mechanism is more applicable to low-fat milk than to other calcium sources. In the United States, milk is likely the most important source of bioavailable calcium because of its frequency of consumption and the ready absorption of calcium, especially in the presence of vitamin D added in fortification. Whereas the suppressive effects of calcium from whole milk may be countered by high intake of vitamin D, a similar reversal of calcium’s effects may not occur with low-fat milk because fat-reduced milk products tend to have a lower vitamin D content (21, 46). Vitamin D, a fat-soluble vitamin, may also be less well absorbed from fat-reduced milk. Although a recent study found similar levels of absorption of vitamin D added to skim and whole milk (47), the quantity used in the study was 25 000 IU in 240 mL milk, substantially above the recommended amount of fortification in the United States of 400 IU/qt (7).

In fact, we found risk was elevated only for low-fat milk and not for whole milk or any other dairy food. When we examined calcium from different food sources, we saw no association for calcium from any source other than low-fat milk. We also saw no association for calcium supplements, although we were able to identify only 151 men who reported such use. In the Physicians’ Health Study (33) as well, the elevated risk of prostate cancer associated with dairy and calcium intake was attributable primarily to intake of skim milk, and calcium intake from skim milk showed a stronger inverse correlation with plasma 1,25-D concentrations than did total daily calcium. Although low-fat milk consumption may be recalled with less error than the consumption of other dairy products, it is unlikely that reporting would be so different between low-fat and whole milk, for example, as to result in complete attenuation of the association for whole milk. Our findings suggest, therefore, an effect attributable primarily to the consumption of low-fat milk, but whether because of its calcium content and vitamin D availability or to another characteristic of low-fat milk is not known. Removal of fat from milk, for example, may remove other components with potentially cancer-protective properties, such as conjugated linoleic acid (48).

Because men of higher socioeconomic status were more likely to drink low-fat milk, detection bias is another possibility. Since 1986, when the US Food and Drug Administration approved the PSA test for monitoring prostate cancer progression and prostate cancer screening, incidence has increased more steeply in men of higher socioeconomic status, who have better awareness of or access to screening modalities (49). Because PSA testing was relatively uncommon before 1991 (43), cases in our sample were more likely to be advanced cases. RR estimates were also largely unchanged when we limited cases to the 107 diagnosed before 1991, when we more conservatively limited cases to the 46 identified before government approval of PSA testing in 1986, and when we controlled for sociodemographic factors that might be linked to screening such as education (49).

Some previous studies suggest a protective effect for phosphorus with adjustment for calcium (32, 34). Phosphorous is found in a variety of food sources, although the principal contributors are milk, meat, poultry, and fish (50). Phosphorus has...
been hypothesized to reduce risk by increasing parathyroid hormone concentrations or reducing calcium bioavailability in the intestine, resulting in higher 1,25D concentrations (32). We saw no effect for phosphorus or evidence of an interaction between calcium and phosphorus.

Although vitamin D is the central factor in the hypothesized mechanism that links calcium to prostate cancer risk, previous studies have not shown a protective effect for dietary vitamin D (32, 34, 35, 41). Some evidence links higher calcium intake with lower concentrations of circulating 1,25-D (33, 41); other studies (51, 52) have noted a protective effect of fatty fish, a principal source of vitamin D. In our cohort, we observed a suggestive protective effect for vitamin D when we combined vitamin D from all dietary sources, analyzed in tertiles, and adjusted for calcium intake. In additional analyses to reduce the potential for inaccurate estimates because of the high collinearity between calcium and vitamin D intake (53), however, vitamin D was no longer associated with risk, whereas the strong, positive association for calcium persisted. Further, even with adjustment for calcium intake, we observed no inverse association with risk of any single food or food group rich in vitamin D, including fish and seafood. Several factors contribute to the difficulty inherent in evaluating the effect of vitamin D effect on risk, including potential error in estimating vitamin D intake (7), the high collinearity between calcium and vitamin D intake, and the importance of both sunlight and diet in determining circulating vitamin D concentrations.

Sunlight has been hypothesized to protect against prostate cancer through 1,25-D production (5). A more recent work also offers evidence that childhood and cumulative, lifetime sun exposure is associated with reduced risk (54). Of the variables related to sunlight exposure that we examined, only adherence to a southern dietary pattern as an adult, possibly reflecting exposure to sunlight that we examined, only adherence to a southern dietary pattern as an adult, possibly reflecting exposure to sunlight, increased risk. Additionally, studies have also shown that individuals who consume a diet rich in vitamin D may have a lower risk of prostate cancer (55). However, there is limited evidence regarding the role of vitamin D in reducing prostate cancer risk.

A primary limitation of the study is that the diet questionnaire used in the 1982–1984 interview was not validated for estimating nutrient intake. Our estimates of calcium and phosphorus intake, however, are similar to independently derived 24-h recall estimates from the USDA 1994 Continuing Survey of Food Intakes by Individuals (\(\bar{x}\) calcium: 750 mg/d; \(\bar{x}\) phosphorus: 1307 mg/d for men aged 51–70 y) (7), and our estimate of vitamin D intake is only slightly higher than an independent estimate, based on 24-h recall data, of 143–148 IU/d for women participating in the 1971–1975 baseline survey of the NHEFS (55). Moreover, with little evidence of systematic bias in estimates, measurement error should generally attenuate associations.

An additional limitation is that, because typical supplement dosage could not be readily estimated from available data, we only considered dietary sources in estimating calcium and vitamin D intake. The small number of men who reported taking calcium (n = 151) or fish oil (n = 50) supplements further limited our ability to evaluate the effect of nondietary sources of these nutrients on disease risk. Finally, our findings were based on only a relatively small number of cases.

As discussed above, an important strength of the study is that, with a follow-up that ended in 1992, cases were less likely to be diagnosed incidentally through PSA testing and more likely to be clinically apparent and advanced, thus allowing for a clearer examination of dietary intake in relation to clinically relevant disease. Other strengths of the NHEFS include its prospective design; relatively long follow-up; excellent ascertainment of cancer outcomes; ethnically, socioeconomically, and geographically diverse population; and detailed diet questionnaire.

In summary, we found that prostate cancer risk was significantly elevated with higher intake of dairy foods and calcium, particularly calcium from low-fat milk. Our findings suggest that dairy intake increases risk of prostate cancer, probably through its calcium content. Reasons for the elevated risk with low-fat milk are unclear, although the reduced content and bioavailability of vitamin D in low-fat milk may play a role. Although 1,25-D has been postulated to reduce risk of prostate cancer and calcium may increase risk by suppressing circulating concentrations of 1,25-D, we failed to see any direct evidence for a protective effect of vitamin D intake in our cohort. Calcium is thought to protect against osteoporosis and colon cancer, and dairy is the primary source of calcium in the US diet. Given the limitations of our findings with respect to recommendations to increase both calcium intake and low-fat milk consumption, the mechanisms by which calcium and low-fat milk might increase prostate cancer risk should be clarified and confirmed to verify that calcium is indeed the critical risk factor.

We thank Ms. Marianne Hyer, Ms. Christine Cox, and Mr. Andrew Balshem for their assistance in bringing together the relevant data.

MT was responsible for analyzing the data and drafting the manuscript. RAB was responsible for identification and confirmation of prostate cancer cases in the dataset and contributed to interpretation of results. BIG contributed to statistical analyses and interpretation of analytic results. RGZ contributed to the original study concept, interpretation of results, and revisions of subsequent drafts of the manuscript, and facilitated acquisition of the dataset for analysis. None of the authors had any conflicts of interest in connection with the research.

REFERENCES

Significant correlations of plasma homocysteine and serum methylmalonic acid with movement and cognitive performance in elderly subjects but no improvement from short-term vitamin therapy: a placebo-controlled randomized study

Catharina Lewerin, Michael Matousek, Gunilla Steen, Boo Johansson, Bertil Steen, and Herman Nilsson-Ehle

ABSTRACT

Background: Deficiencies of vitamin B-12, folic acid, and vitamin B-6—as defined by laboratory measures—occur in 10–20% of elderly subjects. The clinical significance remains unresolved.

Objective: The objective was to explore any association between vitamin status and vitamin treatment and movement and cognitive performance in elderly subjects.

Design: Community-dwelling subjects (n = 209) with a median age of 76 years were randomly assigned to daily oral treatment with 0.5 mg cyanocobalamin, 0.8 mg folic acid, and 3 mg vitamin B-6 or placebo (double blind) for 4 months. Movement and cognitive performance tests were performed before and after treatment.

Results: A high plasma total homocysteine (tHcy) concentration (≥16 μmol/L) was found in 64% of men and in 45% of women, and a high serum methylmalonic acid (MMA) concentration (≥0.34 μmol/L) was found in 11% of both sexes. Movement time, digit symbol, and block design (adjusted for age, sex, smoking, and creatinine) correlated independently with plasma tHcy (P < 0.01, < 0.05, and < 0.01, respectively); the simultaneity index and block design correlated with serum MMA (P < 0.05 for both). Vitamin therapy significantly decreased plasma tHcy (32%) and serum MMA (14%). No improvements were found in the movement or cognitive tests compared with placebo. Neither vitamin therapy nor changes in plasma tHcy, serum MMA, serum vitamin B-12, plasma folate, or whole-blood folate correlated with changes in movement or cognitive performance.

Conclusions: High plasma tHcy and serum MMA were prevalent and correlated inversely with movement and cognitive performance. Oral B vitamin treatment normalized plasma tHcy and serum MMA concentrations but did not affect movement or cognitive performance. This might have been due to irreversible or vitamin-independent neurocognitive decline or to an insufficient dose or duration of vitamins.

KEY WORDS Elderly, homocysteine, methylmalonic acid, cognition, movement, controlled trial

INTRODUCTION

The clinical significance of vitamin B-12, folic acid, and vitamin B-6 deficiency, as defined by laboratory measures, is unresolved (1–3). Correlations between low vitamin status and poor cognitive function have been found in community-dwelling elderly subjects (4–9), prospective community-based studies (10–12), and neuropsychiatric patients (13–17). Some open studies have shown correlations between vitamin supplementation and cognitive improvement (18–21), whereas double-blind placebo-controlled studies diverge in outcome (22, 23).

Total plasma homocysteine (tHcy) concentrations are elevated in Parkinson disease (24), a disorder that also carries an increased risk of depression (25) and other mental disturbances (26). L-Dopa can cause hyperhomocysteinemia in Parkinson disease patients and the extent is influenced by B vitamin status (27). Gait abnormalities in elderly nondemented subjects have been found to be a significant predictor of non-Alzheimer dementia (28). Plasma tHcy correlated with a subsequent decline in physical functioning during 3 years in subjects aged 70–79 years (29).

Reasons for the neurocognitive impairment from vitamin B-12, folic acid, and vitamin B-6 deficiencies are an inadequate supply of methyl groups, DNA damage, and premature apoptosis caused by disturbed methionine and folate metabolism (30, 31). Furthermore, homocysteine is toxic to neurons (32–34), vascular endothelial cells (35), and connective tissue (36).

Normalization of laboratory vitamin status in the elderly may be accomplished with oral vitamins but the clinical significance remains unclear. The relations between vitamin status (including related metabolites) and cognitive and movement performance in the elderly have, to our knowledge, not yet been investigated. Furthermore, the diagnosis of clinical vitamin deficiency is controversial.

The aims of the present study were to investigate, in an elderly population, any association between movement and cognitive...
performance and vitamin status and whether treatment with therapeutic doses of vitamins B-12, folic acid, and vitamin B-6 improved this clinical performance.

SUBJECTS AND METHODS

Subjects

The total study group comprised 209 community-dwelling men and women with a mean age of 76 y and 5 mo (37). At baseline, all subjects underwent cognitive testing. One hundred ninety-five persons (117 women and 78 men) were also investigated with the Postural-Locomotor-Manual (PLM) test (Table 1). Of these 195 subjects, 126 were randomly assigned to receive vitamin therapy and 69 to receive placebo. The vitamin and placebo groups were well balanced with respect to baseline laboratory tests, movement and cognitive performance, and medication use for neurologic and cardiovascular disorders (Table 1). Subjects in the vitamin group received a daily tablet containing 500 µg cyanocobalamin, 800 µg folic acid, and 3 mg vitamin B-6 hydrochloride (manufactured and supplied by Recip AB, Årsta, Sweden), and all subjects in the placebo group received an identical (other than the vitamin content) placebo tablet. The duration of the intervention was 4 mo. To ensure compliance, all subjects received a specified blinded number of tablets, and at the end of the study, the number of remaining tablets was compared with the initial number and planned intake during the study period. Informed consent was obtained from all probands, and the Research Ethics Committee of the Medical Faculty of Göteborg University approved the protocol.

Postural-Locomotor-Manual test

Movement performance (n = 195) was measured with a Postural-Locomotor-Manual (PLM) test, a noninvasive optoelectronic technique using infrared light (Qualisys AB, Göteborg, Sweden). The PLM test (38) consists of a complex motion during which the patient moves an object from the floor 1.5 m forward and positions it on a stand at the height of their chin (Figure 1). Six reflective markers are placed on the right side of the head, shoulder, elbow, hip, ankle, and left foot of each subject. The seventh marker is placed on the test object, a metal handle fastened to a cylindrical horizontal plate weighing 550 g. A camera system registers the infrared light pulses reflected from the markers. The position of the markers is calculated 50 times/s as 2-dimensional (x, y) Cartesian room coordinates and is stored in a computer. The coordinate data are processed by using commercially available software, the PLM program. The time taken for each subject to direct active arm movement to lift up and place the object on the stand (maneuver phase; M phase) were calculated. The overlap

### Table 1

Observations in the vitamin and placebo groups at the start of the study

<table>
<thead>
<tr>
<th></th>
<th>Vitamin group (n = 126)</th>
<th>Placebo group (n = 69)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>75.7 ± 4.7</td>
<td>75.6 ± 4.0</td>
<td>NS</td>
</tr>
<tr>
<td>Men (%)</td>
<td>38</td>
<td>44</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.8 ± 3.2</td>
<td>25.1 ± 3.8</td>
<td>0.04</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>14</td>
<td>9</td>
<td>NS</td>
</tr>
<tr>
<td>Dropouts (%)</td>
<td>13</td>
<td>16</td>
<td>NS</td>
</tr>
<tr>
<td>Use of cardiovascular medication (%)</td>
<td>47</td>
<td>49</td>
<td>NS</td>
</tr>
<tr>
<td>Use of antiepileptics, neuroleptics, or antidepressants (%)</td>
<td>22</td>
<td>23</td>
<td>NS</td>
</tr>
<tr>
<td>PLM test (s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Movement time</td>
<td>2.1 ± 0.6</td>
<td>2.1 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Postural phase</td>
<td>0.9 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Locomotor phase</td>
<td>1.6 ± 0.4</td>
<td>1.5 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>Manual phase</td>
<td>1.4 ± 0.4</td>
<td>1.4 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Simultaneity index</td>
<td>1.8 ± 0.2</td>
<td>1.9 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Cognitive test (score)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digit span forward</td>
<td>5.8 ± 1.1</td>
<td>5.9 ± 1.2</td>
<td>NS</td>
</tr>
<tr>
<td>Digit span backward</td>
<td>4.5 ± 1.2</td>
<td>4.6 ± 1.0</td>
<td>0.11</td>
</tr>
<tr>
<td>Identical forms</td>
<td>23.2 ± 7.7</td>
<td>24.6 ± 7.6</td>
<td>NS</td>
</tr>
<tr>
<td>Visual reproduction</td>
<td>6.9 ± 3.0</td>
<td>7.0 ± 2.9</td>
<td>NS</td>
</tr>
<tr>
<td>Synonyms</td>
<td>22.4 ± 4.8</td>
<td>22.6 ± 4.8</td>
<td>NS</td>
</tr>
<tr>
<td>Block design</td>
<td>18.3 ± 6.3</td>
<td>19.8 ± 7.2</td>
<td>0.18</td>
</tr>
<tr>
<td>Digit symbol</td>
<td>35.4 ± 9.9</td>
<td>37.8 ± 11.4</td>
<td>0.18</td>
</tr>
<tr>
<td>Thurstone’s picture memory test</td>
<td>20.5 ± 4.5</td>
<td>21.0 ± 3.9</td>
<td>NS</td>
</tr>
<tr>
<td>Figure classification</td>
<td>15.8 ± 4.6</td>
<td>16.6 ± 4.6</td>
<td>NS</td>
</tr>
<tr>
<td>Serum measurements</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B-12 (pmol/L)</td>
<td>305 ± 130</td>
<td>359 ± 198</td>
<td>0.06</td>
</tr>
<tr>
<td>Methylmalonic acid (µmol/L)</td>
<td>0.22 ± 0.1</td>
<td>0.22 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>101 ± 15.9</td>
<td>101 ± 18.8</td>
<td>NS</td>
</tr>
<tr>
<td>Iron (µmol/L)</td>
<td>15.8 ± 4.5</td>
<td>15.8 ± 4.3</td>
<td>NS</td>
</tr>
<tr>
<td>Iron-binding capacity (µmol/L)</td>
<td>56.3 ± 6.5</td>
<td>55.0 ± 7.1</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma measurements</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folate (nmol/L)</td>
<td>15.7 ± 6.1</td>
<td>16.4 ± 5.1</td>
<td>NS</td>
</tr>
<tr>
<td>Total homocysteine (µmol/L)</td>
<td>17.8 ± 5.5</td>
<td>16.1 ± 4.5</td>
<td>0.07</td>
</tr>
</tbody>
</table>

* Age and sex-adjusted P values (n = 195). Comparisons between groups made according to the method of O’Brien (48).

* Unadjusted ± SD (all such values)
of the different phases is illustrated by using the simultaneity index (SI), as calculated from the sum of the P, L, and M phase durations divided by the MT: SI = (P + L + M)/MT. A high SI value (near 2.0) indicated good coordination of the P, L, and M phases into a smooth and efficient body movement, whereas a value approaching 1.0 represented poor motor coordination with more sequential performance of the phases. Each subject performed 5 PLM trials; the last 2 were analyzed, and the fastest was used for further analyses. Data were selected from a representative file according to a previously described method (38–41).

The variables chosen to represent the PLM test in statistical analyses were MT, which is an indicator of the overall movement performance in this test, and the SI, which measures the coordination of different parts (ie, phases) of the motor act.

Cognitive tests

Cognitive testing was conducted by the same psychologist (GS) at baseline and after 4 mo. A comprehensive battery of cognitive tests was administered to characterize the overall level of cognitive abilities among the probands. Testing took ≈1 h. All tests except the memory test had time limits. Test scores were equal to the number of correct responses, except for figure grouping and identical forms, where corrections were made for guesses. In the analysis directed toward potential relations across markers of vitamin status and movement performance we focused on tests measuring psychomotor ability and mental speed, eg, the digit symbol test and block design. The following tests were used on the 2 occasions:

- digit span forward/backward, which measures short-term memory. The subjects have to reproduce a series of digits, which increase gradually. In the backward version, the subjects have to repeat the digits backward. The maximum (best) score is 9 in the forward and 8 in the backward subtests (42, 43).
- identical forms, which measures perceptual speed. This test contains 60 items of identification. For each item, a complex figure is compared with 5 other figures, and the one that is identical is marked. The maximum (best) score is 30 (45).
- visual reproduction, which is a measure of visual memory. In this test 4 drawings are shown to the tested subject to be remembered and reproduced. The function is dependent on the memory for visuospatial relations but also to some extent to motor functions. The scoring followed the Wechsler Memory Scale (42). The maximum (best) score is 14.
- synonyms, which measures verbal ability. The subjects have to select from among 5 words a synonym for a given word. The maximum (best) score is 30 (44).

- block design, which measures spatial ability. This test consists of 7 designs that have to be made out of red, white, and red and white blocks. The maximum (best) score is 42. Bonuses are given for rapid performances (43, 44).
- digit symbol, which is a test of perceptual speed with a time limit of 90 s. The subjects are asked to replace digits with symbols according to an existing code. This presumes concentration, sustained attention, learning, visual-motor coordination, and cognitive flexibility. This test has also been used as a biomarker of aging in many studies. The maximum (best) score is 90 (43).

- Thurstone’s Picture Memory Test, which measures long-term memory. The subjects look at 28 pictures consecutively, which are presented at a rate of every 5 s; they are later asked to identify the picture among 4 similar pictures. The pictures were enlarged to minimize problems due to visual impairments in the subjects. The maximum (best) score is 28 (44, 45).
- figure classification, which measures inductive reasoning. In each item, 5 figures are given. The figure that is different from the others is to be marked. The maximum (best) score is 30 (45).

Blood sampling and laboratory methods

Blood samples were collected at the start of the study and after 1 and 4 mo. Samples were obtained with the subjects in a recumbent position, after an overnight fast. Laboratory methods are described in detail elsewhere (37). Serum methylmalonic acid (MMA) was measured by using capillary gas chromatography and mass spectrometry (46). Plasma tHcy was measured by using HPLC with fluorescence detection (47). The current health-related upper reference limits for routine clinical use by the laboratory were 16 μmol/L for plasma tHcy and 0.34 μmol/L for serum MMA, defined as the 97.5% percentile of values of blood donors and healthy persons aged 20–60 y (no age or sex difference).

Statistical analysis

In testing for differences between 2 groups, a method according to O’Brien was used (48), which provided a means of analyzing differences in not only the mean values (location) but also in distribution, ie, location as well as shape and SD. Partial correlation coefficients, adjusted for age and sex, were calculated between vitamin status and PLM and between vitamin status and cognitive performance, respectively. Multiple regression analyses, adjusted for age, sex, smoking habits, and serum creatinine were conducted for selected PLM and cognitive variables. In addition, stepwise multiple regression analysis with both demographic and laboratory variables as possible explanatory variables was performed. Possible effects of vitamin treatment were analyzed with a pairwise test of change within each group, which was followed by a test of differences in mean change between both groups. Two-tailed tests were used throughout, and a significance level of P < 0.05 was considered statistically significant. Non-Gaussian distributions were log transformed. The software used was part of a statistical program system developed at the Department of Geriatric Medicine, Göteborg University.
RESULTS

The study population was described in terms of the numbers in each test group and the reduction in numbers of the total study group (Figure 2). The number of participants in both the PLM and cognitive tests was 195 at start of the study and 171 after 4 mo. At the end of the study, 162 participants could be evaluated with the PLM tests, 179 with the cognitive tests, and 161 with both tests. Dropouts and excluded subjects (n = 48) were slightly older than and had longer MTs and L phases than did the remaining participants (n = 161). There were no significant differences in cognitive performance and laboratory values between the groups (data not shown).

Univariate analysis at baseline

High plasma tHcy concentrations, as defined by laboratory reference intervals (≥16 μmol/L), were found in 64% of the men and in 45% of the women; high serum MMA (≥0.34 μmol/L) concentrations were found in 11% of both sexes (37). Serum MMA, plasma folate, whole-blood folate, and serum vitamin B-12 correlated significantly with plasma tHcy, but serum vitamin B-12 did not correlate with serum MMA (Table 2). Four of 5 PLM components correlated with plasma tHcy and 2 with serum MMA. Seven of 9 components of the cognitive performance tests correlated with plasma tHcy and 3 with serum MMA. Further multivariate regression models, including sex, smoking habits, blood hemoglobin, erythrocyte mean cell volume, whole-blood folate, serum creatinine, serum vitamin B-12, plasma folate, transferrin saturation, and anamnestic and laboratory exclusion criteria indicating a nonhealthy state (37), were also performed (data not shown). Significant correlations were found for the same variables, except for block design versus serum MMA. In this extensive multivariate analysis, inferior performance in all 4 dependent variables correlated independently with age and in 3 (digit symbol, block design, and MT) with plasma tHcy. Male sex correlated with superior performance in MT and block design.

Multivariate analysis at baseline

Multivariate analyses, adjusted for age, sex, smoking habits, and serum creatinine, were performed with 2 movement and 2 cognitive variables as dependent variables (Table 2). MT correlated with plasma tHcy, SI with serum MMA, digit symbol with plasma tHcy, and block design with plasma tHcy and serum MMA. Further multivariate regression models, including sex, smoking habits, blood hemoglobin, erythrocyte mean cell volume, whole-blood folate, serum creatinine, serum vitamin B-12, plasma folate, transferrin saturation, and anamnestic and laboratory exclusion criteria indicating a nonhealthy state (37), were also performed (data not shown). Significant correlations were found for the same variables, except for block design versus serum MMA. In this extensive multivariate analysis, inferior performance in all 4 dependent variables correlated independently with age and in 3 (digit symbol, block design, and MT) with plasma tHcy. Male sex correlated with superior performance in MT and block design.
Intervention study

Plasma tHcy and serum MMA

Mean plasma tHcy values decreased by 32% and mean serum MMA by 14% in vitamin-treated subjects. These significant changes resulted in a distribution of these values similar to those of younger healthy subjects (37).

Movement and cognitive function

The mean time span for the PLM tests became somewhat shorter in both groups after 4 mo, but the differences between the vitamin and the placebo groups were not significant (Table 4). Improvements in the cognitive tests were observed in both groups. For identical forms and synonyms, the mean scores of the

TABLE 3

Multiple regression model (in the total study group) of the correlation between movement time, simultaneity index, digit symbol, block design, and laboratory variables at the start of the study

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>n</th>
<th>Explanatory variable</th>
<th>Regression coefficient (B)</th>
<th>R²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Movement time</td>
<td>194</td>
<td>Serum MMA</td>
<td>0.68</td>
<td>0.13</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>195</td>
<td>Plasma tHcy</td>
<td>0.024</td>
<td>0.15</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>195</td>
<td>Plasma folate</td>
<td>−0.0054</td>
<td>0.12</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>195</td>
<td>Whole-blood folate</td>
<td>−0.000022</td>
<td>0.12</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>195</td>
<td>Serum vitamin B-12</td>
<td>−0.00032</td>
<td>0.12</td>
<td>NS</td>
</tr>
<tr>
<td>Simultaneity index</td>
<td>194</td>
<td>Serum MMA</td>
<td>−0.29</td>
<td>0.11</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>195</td>
<td>Plasma tHcy</td>
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<td>0.09</td>
<td>NS</td>
</tr>
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<td></td>
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<td>NS</td>
</tr>
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<td>NS</td>
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<td></td>
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<td>Serum vitamin B-12</td>
<td>0.000070</td>
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</tr>
<tr>
<td>Block design</td>
<td>206</td>
<td>Serum MMA</td>
<td>−12.21</td>
<td>0.16</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
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<td>Plasma tHcy</td>
<td>−0.31</td>
<td>0.17</td>
<td>&lt;0.01</td>
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<td></td>
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<td>Plasma folate</td>
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<td>0.13</td>
<td>NS</td>
</tr>
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<td>Whole-blood folate</td>
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<td>NS</td>
</tr>
<tr>
<td></td>
<td>207</td>
<td>Serum vitamin B-12</td>
<td>0.0028</td>
<td>0.13</td>
<td>NS</td>
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<tr>
<td>Digit symbol</td>
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<td>Serum MMA</td>
<td>−11.08</td>
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<td>0.083</td>
<td>0.10</td>
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<tr>
<td></td>
<td>204</td>
<td>Whole-blood folate</td>
<td>−0.004</td>
<td>0.10</td>
<td>NS</td>
</tr>
</tbody>
</table>

1 Values were adjusted for age, sex, smoking habits, and serum creatinine. See Table 1 for units. MMA, methylmalonic acid; tHcy, total homocysteine.
placebo group increased more than did those of the vitamin group, and these differences were significant. The univariate correlations at baseline between serum MMA and plasma tHcy and movement and cognitive performance remained significant after treatment in both the placebo- and vitamin-treated groups (data not shown). Neither basal plasma tHcy, serum MMA, serum vitamin B-12, plasma and whole-blood folate nor changes in these components during the treatment period or vitamin therapy per se showed any associations with change in movement or cognitive performance.

**DISCUSSION**

In this population of community-dwelling elderly subject, deficiency of vitamin B-12 was observed in 7.2% and of folic acid in 11%. High plasma tHcy concentrations were common (64% in men, 45% in women), and high serum MMA concentrations were present in 11% of the population (37). Almost all cognitive tests correlated inversely with plasma tHcy before treatment. These results are consistent with previous findings (4, 9, 12, 13). In addition, we found correlations between both movement and cognitive performance on the one hand and serum MMA on the other. However, the influence of vitamin concentrations on performance in the total study population was limited. This is consistent with some reports (4, 12), but conflicts with others (5, 49). These discrepancies are presumably due to differences between populations with regard to health status, sex, and age and to methodologic differences.

Multivariate analyses showed significant and independent correlations between movement and cognitive performance and plasma tHcy and serum MMA concentrations. A novel finding in this study was the indication of different significances of metabolite levels for different aspects of movement and cognitive performance (Table 3).

MT and decreased mobility, in both legs and arms (L and M phases), correlated with plasma tHcy. Slow MT and poor coordination of the motor act (SI) correlated with serum MMA. MT in the PLM test was previously shown to be associated with vascular disease, brain atrophy, and cerebral white matter lesions in the elderly (40, 41, 50). SI deteriorates with age (40), in Parkinson disease (51), and in normal-pressure hydrocephalus (52). The speed (MT) and coordination (SI) of the motor function in many Parkinson disease patients have been shown to indicate deficient functioning of certain brain regions, eg, basal ganglia (51, 52). It is noteworthy that the SI was able to measure the coordinative motor capacity in the brain in these patients. Whether movement deterioration, which resembles early Parkinsonism (53, 54) and was seen in this study, is preventable with vitamin supplementation needs to be confirmed in controlled trials. The associations between plasma tHcy and movement performance have not been extensively investigated. In a longitudinal study, a decline in physical function over a 3-y interval was essentially unchanged after the intervention. This finding

**TABLE 4**

Movement and cognitive performance at baseline, mean changes after treatment, and *P* values for differences in mean changes between the vitamin and placebo groups

<table>
<thead>
<tr>
<th></th>
<th>Vitamin group</th>
<th>Placebo group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>n</em></td>
<td>Before treatment</td>
</tr>
<tr>
<td>Movement (s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Movement time</td>
<td>105</td>
<td>2.08 ± 0.6</td>
</tr>
<tr>
<td>Postural phase</td>
<td>105</td>
<td>0.91 ± 0.2</td>
</tr>
<tr>
<td>Locomotor phase</td>
<td>105</td>
<td>1.53 ± 0.4</td>
</tr>
<tr>
<td>Manual phase</td>
<td>105</td>
<td>1.37 ± 0.4</td>
</tr>
<tr>
<td>Simultaneity index</td>
<td>105</td>
<td>1.86 ± 0.2</td>
</tr>
<tr>
<td>Cognitive test (score)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLM test (s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digit span forward</td>
<td>115</td>
<td>5.8 ± 1.1</td>
</tr>
<tr>
<td>Identical forms</td>
<td>115</td>
<td>2.33 ± 7.6</td>
</tr>
<tr>
<td>Syntonyms</td>
<td>110</td>
<td>6.9 ± 3.1</td>
</tr>
<tr>
<td>Block design</td>
<td>114</td>
<td>18.5 ± 6.3</td>
</tr>
<tr>
<td>Digit symbol</td>
<td>113</td>
<td>35.1 ± 10.0</td>
</tr>
<tr>
<td>Thurmrose’s picture memory test</td>
<td>115</td>
<td>20.3 ± 4.8</td>
</tr>
<tr>
<td>Figure classification</td>
<td>113</td>
<td>15.8 ± 4.8</td>
</tr>
</tbody>
</table>

1 PLM, Postural-Locomotor-Manual. There were no significant differences in baseline values between the vitamin and placebo groups.
2 All values are *x* ± SD.
3 All values are *x* ± SEM.
4 Two-sample *t* test of the differences in mean change between the vitamin and placebo groups.

---

**Notes:**
- *LEWERIN ET AL* 1160
- **TABLE 4** Movement and cognitive performance at baseline, mean changes after treatment, and *P* values for differences in mean changes between the vitamin and placebo groups.
- MT: Movement Time
- SI: Simultaneity Index
- *P* values were calculated using a two-sample *t* test for differences in mean change between the vitamin and placebo groups.
indicates a lack of significant clinical response to vitamin therapy in the present study.

The vitamin doses were chosen to treat pronounced deficiencies of these vitamins. The vitamin B-12 dose of 0.5 mg was 250 times the current US recommended daily allowance (55) and, as for the dose of folic acid, is considered adequate (56–59). Correlations were found between duration of cognitive symptoms and neurologic symptoms and response to therapy (20, 60). In a placebo-controlled study, the vitamin doses used were lower than those in the present study but were given for 12 mo instead of 4 mo and led to significant improvements in almost all cognitive tests (23). The duration of both symptoms and therapy might also be important in this community-dwelling, essentially nondemented, and mobile population. Instead of analyzing the group in terms of men and women, we adjusted for sex in the analyses. A possible differential role of vitamin status in women and men needs to be studied in larger sample sizes.

The short duration of the intervention and the relatively small sample size were limitations of the study. Its strengths were the use of an optoelectronic method, which enabled an objective and precise measurement of the subject’s mobility of the upper and lower extremities and of movement coordination, and the comprehensive cognitive test battery. Further studies are needed to investigate the importance of the different B vitamins for various neurocognitive functions.

In summary, high concentrations of plasma tHcy and serum MMA were more common than were actual vitamin B deficiencies in this population of community-dwelling elderly subjects. Plasma tHcy and serum MMA correlated independently and differently with movement and cognitive performance, which suggests different pathophysiologic mechanisms. However, 4 mo of oral vitamin treatment normalized plasma tHcy and serum MMA but failed to improve movement and cognitive performance. This result might be attributable to irreversible or vitamin-independent neurocognitive decline or to an insufficient dosage of vitamins or duration of vitamin treatment.

We thank V Sundh for statistical advice and support. BS and HN-E were the principal designers of the study, MM performed and analyzed the PLM test. GS performed and analyzed the cognitive tests. CL gathered data and was responsible for the statistical calculations and for the preliminary preparation of the manuscript. All authors contributed to the scientific workup and the revision of the manuscript. None of the authors had a personal or financial conflict of interest with respect to this study.

REFERENCES


Association of diet with serum insulin-like growth factor I in middle-aged and elderly men

Susanna C Larsson, Katarina Wolk, Kerstin Brismar, and Alicja Wolk

ABSTRACT

Background: Insulin-like growth factor I (IGF-I) has been implicated in several chronic diseases, including cancer, heart disease, and osteoporosis.

Objective: Our aim was to assess whether intakes of total energy, alcohol, vitamins, minerals, and foods rich in protein and minerals (including red meat, fish and seafood, poultry, and milk) are associated with serum IGF-I concentrations in middle-aged and elderly men.

Design: We measured serum IGF-I concentrations in 226 free-living healthy men aged 42–76 y. The average of fourteen 24-h dietary telephone interviews performed over 1 y was used to estimate long-term dietary intake.

Results: We observed statistically significant positive associations between intakes of protein (P for trend = 0.001) and zinc (P for trend = 0.002) and serum IGF-I concentrations after adjusting for age. The difference in mean IGF-I concentrations for the highest compared with the lowest quintile of intake was ≈17% (162 μg/L compared with 139 μg/L) for protein and ≈16% (166 μg/L compared with 143 μg/L) for zinc. Consumption of red meat (P for trend = 0.05) and fish and seafood (P for trend = 0.07) was modestly positively associated with IGF-I concentrations. Other dietary factors were not associated with IGF-I concentrations.

Conclusion: In this population of healthy well-nourished men, greater dietary intakes of protein, zinc, red meat, and fish and seafood were associated with higher IGF-I concentrations. Other dietary factors were not associated with IGF-I concentrations.

SUBJECTS AND METHODS

Study population

Details of this study have been described elsewhere (21). Briefly, the study population for this investigation was 226 free-living healthy men aged 42–76 y, who were randomly selected from the population register of Uppsala (city) and nearby (countryside) in central Sweden. The men participated in fourteen 24-h dietary telephone interviews performed over 1 y. We obtained blood samples within 2 wk after completion of the last interview. The ethical committees at the Uppsala University Hospital and the Karolinska Institutet in Stockholm approved this study.

Measurements

Data were collected on biochemical measurements of blood, anthropometric measurements, cigarette smoking status, and dietary intakes. Blood samples were drawn in the morning after

1 From the Division of Nutritional Epidemiology, The National Institute of Environmental Medicine (SCL and AW) and the Departments of Medicine (KW) and Molecular Medicine (KB), Unit of Endocrinology and Diabetes, Karolinska Institutet, Stockholm.

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3 Address reprint requests to SC Larsson, Division of Nutritional Epidemiology, The National Institute of Environmental Medicine, Karolinska Institutet, PO Box 210, SE-171 77 Stockholm, Sweden. E-mail: susanna.larsson@imm.ki.se.

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a 12-h overnight fast. Serum was separated by centrifugation at 1200 × g for 10 min at 20 °C after blood samples were held at room temperature for 2 h. Sera were stored at −70 °C until analyzed. Serum IGF-I was measured by radioimmunoassay after acid ethanol extraction (22). The sensitivity of the assay was 2 μg/L and the within- and between-assay CVs for IGF-I were 4% and 11%, respectively. Insulin-like growth factor binding protein (IGFBP)-1 was determined by radioimmunoassay according to the method of Povoa et al (23). The antibodies used were raised in rabbits against purified human amniotic protein, and the cross-reaction with IGFBP-2 and IGFBP-3 was <0.1%.

The sensitivity of the assay was 3 μg/L, and the within- and between-assay CVs for IGF-I were 3% and 10%, respectively. There was no significant cross-reactivity between IGF-I, IGF-II, or other peptide hormones with IGF-I antiserum.

Body mass index (BMI) was calculated by dividing body weight (in kg) by the square of height (in m). Waist was measured with the subjects in the supine position. The abdominal sagittal circumference was recorded at the umbilical level as the height of the abdomen measured from the examination couch when lying down with the legs straight.

**Dietary assessment**

We used the average of fourteen 24-h dietary telephone interviews conducted over 1 y (about once per month) to estimate long-term dietary intake. The research dietician who performed the telephone interviews used a standardized 24-h diet recall technique completed with probing questions. Portion sizes were described in household measures. An administrative program had been choosing random days for consecutive dietary interviews for each participant, covering all weekdays as well as weekends. The interviews were entered by using a personal computer nutrient software package MATS (24). Intakes of nutrients were calculated by using the Swedish Food Administration Food Database (25), which includes 1593 foods and dishes. For dishes reported but not included in this database, the dietician obtained recipes from the participants and entered appropriate amounts of the component foods.

**Statistical analyses**

We first calculated the means (± SDs) and proportions of covariates for this sample of men. Spearman correlation coefficients were calculated between age and other nondietary variables and IGF-I. Because the distribution of serum IGFBP-1 concentrations was positively skewed, we conducted a logarithmic transformation of this variable to improve normality.

The dietary factors considered in the present analysis were intakes of total energy, alcohol, macronutrients, vitamins (including vitamins A, B-6, C, D, and E; thiamine; riboflavin; and folate) minerals (including calcium, magnesium, potassium, and zinc), and foods rich in protein and minerals (including red meat, fish and seafood, poultry, and total milk). All intakes of nutrients except alcohol intake were energy adjusted by using the residual method (26). Each dietary variable (except poultry because of limited range of consumption) was treated as categorical (by quintiles) and continuous. We used generalized linear regression models to estimate mean IGF-I concentrations by quintiles of each dietary variable. Regression models were used to determine the differences in IGF-I concentrations that corresponded to 2-SD differences in dietary intake. All analyses were controlled for age (as a continuous variable). Adjustment for anthropometric variables (including weight, height, BMI, sagittal measure, and waist), smoking, and serum IGFBP-1 concentrations did not change the results; therefore, these variables were not included in the final models. We used the SAS software (version 8.2; SAS Institute Inc, Cary, NC) for analyses. All P values were two-sided; P < 0.05 was considered statistically significant.

**RESULTS**

For the 226 men included in this analysis, the mean age was 60.5 ± 10.1 y and the mean serum IGF-I concentration was 147.5 ± 40.9 μg/L (Table 1). Their reported energy intake was 2092 ± 451 kcal/d. Age [Spearman correlation coefficient (r) = −0.44, P < 0.0001] and serum IGFBP-1 (r = −0.31, P < 0.0001) were inversely correlated with serum IGF-I concentrations. None of the anthropometric variables examined, including height, weight, BMI, waist, and sagittal measure, was significantly correlated with IGF-I concentrations (data not shown).

The associations between intakes of energy, alcohol, macronutrients, and micronutrients and serum IGF-I concentrations are shown in Table 2. In the age-adjusted regression analysis, protein intake was positively associated with IGF-I concentrations (β = 16.0 for 2-SD difference, P for trend = 0.001). Serum IGF-I concentrations were ≈17% higher in men in the top quintile of protein intake than in men in the bottom quintile (P for difference between extreme quintiles = 0.005). We observed a positive association between potassium (β = 11.0 for 2-SD difference, P for trend = 0.03) and zinc (β = 15.3 for 2-SD difference, P for trend = 0.002) and serum IGF-I. However, potassium and zinc were correlated (Spearman correlation coefficient r = 0.41, P < 0.0001), and, when both nutrients were included in the same model, only zinc intake remained significantly related to IGF-I. Men in the highest quintile of zinc intake had ≈16% higher serum IGF-I concentrations than men in the lowest quintile (P for

---

**Table 1**

Characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>60.5 ± 10.12</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.3 ± 3.2</td>
</tr>
<tr>
<td>IGF-I (μg/L)</td>
<td>147.5 ± 40.9</td>
</tr>
<tr>
<td>IGFBP-1 (μg/L)</td>
<td>26 (3–93)4</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>21</td>
</tr>
<tr>
<td>Dietary intake</td>
<td></td>
</tr>
<tr>
<td>Total energy (kcal/d)</td>
<td>2092 ± 451</td>
</tr>
<tr>
<td>Alcohol (g/d)</td>
<td>11.6 ± 11.8</td>
</tr>
<tr>
<td>Total fat (g/d)</td>
<td>79.2 ± 9.5</td>
</tr>
<tr>
<td>Carbohydrate (g/d)</td>
<td>249 ± 28</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>75.7 ± 8.6</td>
</tr>
<tr>
<td>Calcium (mg/d)</td>
<td>976 ± 233</td>
</tr>
<tr>
<td>Magnesium (mg/d)</td>
<td>328 ± 40</td>
</tr>
<tr>
<td>Potassium (mg/d)</td>
<td>3201 ± 461</td>
</tr>
<tr>
<td>Zinc (mg/d)</td>
<td>10.4 ± 1.3</td>
</tr>
</tbody>
</table>

1 n = 226. IGF-I, insulin-like growth factor I; IGFBP-1, insulin-like growth factor binding protein 1.
2 ± SD (all such values).
3 The distribution was positively skewed.
4 Median; range in parentheses.
5 All nutrients except alcohol were energy-adjusted by using the residual method (26).
Serum IGF-I concentrations were positively associated with IGF-I concentrations, although fish and seafood (g/d) was modestly positively associated with IGF-I. We examined many dietary factors; therefore, some observed associations were positive, but not statistically significant. We observed that protein and zinc were significant determinants of serum IGF-I concentrations, although only the association with red meat was statistically significant. Serum IGF-I concentrations were 13% higher in the highest quintile than in the lowest quintile of red meat consumption ($P$ for trend between extreme quintiles = 0.03). The results did not change when we included red meat, fish and seafood, poultry, and milk simultaneously in 1 model (data not shown).

**DISCUSSION**

In this population-based study of healthy well-nourished men, we observed that protein and zinc were significant determinants of serum IGF-I concentrations. In addition, consumption of red meat and fish and seafood, which are high in protein and minerals, was modestly positively associated with IGF-I. We examined many dietary factors; therefore, some observed associations were positive, but not statistically significant. We observed that protein and zinc were significant determinants of serum IGF-I concentrations, although only the association with red meat was statistically significant. Serum IGF-I concentrations were 13% higher in the highest quintile than in the lowest quintile of red meat consumption ($P$ for trend between extreme quintiles = 0.03). The results did not change when we included red meat, fish and seafood, poultry, and milk simultaneously in 1 model (data not shown).

### TABLE 2

Age-adjusted mean serum insulin-like growth factor I (IGF-I) concentrations (μg/L) with 95% CIs by quintiles of total energy, alcohol, macronutrient, and mineral intakes and the differences in serum IGF-I concentrations for 2-SD differences in the dietary variable.

<table>
<thead>
<tr>
<th>Quintile of dietary intake</th>
<th>2-SD difference in intake</th>
<th>β (95% CI)</th>
<th>P for β</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Low)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-I</td>
<td>147 (136, 158) +</td>
<td>154 (143, 165)</td>
<td>149 (131, 154)</td>
</tr>
<tr>
<td>Alcohol (g/d)</td>
<td>1.8–5.0 [46]</td>
<td>7.6–8.16 [45]</td>
<td>8.17–8.70 [45]</td>
</tr>
<tr>
<td>Total fat (g/d)</td>
<td>72.3–76.8 [45]</td>
<td>76.9–81.6 [45]</td>
<td>81.7–87.0 [45]</td>
</tr>
<tr>
<td>IGF-I</td>
<td>147 (136, 158)</td>
<td>151 (139, 162)</td>
<td>149 (138, 160)</td>
</tr>
<tr>
<td>IGF-I</td>
<td>148 (137, 159)</td>
<td>141 (130, 152)</td>
<td>149 (138, 160)</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>&lt;68.6 [46]</td>
<td>68.6–7.77 [46]</td>
<td>73.7–8.70 [46]</td>
</tr>
<tr>
<td>Calcium (mg/d)</td>
<td>139 (129, 150)</td>
<td>145 (134, 156)</td>
<td>154 (139, 162)</td>
</tr>
<tr>
<td>Magnesium (mg/d)</td>
<td>772 [45]</td>
<td>772–901 [46]</td>
<td>901–1026 [45]</td>
</tr>
<tr>
<td>Potassium (mg/d)</td>
<td>145 (134, 156)</td>
<td>145 (134, 156)</td>
<td>147 (136, 158)</td>
</tr>
<tr>
<td>Zinc (mg/d)</td>
<td>&lt;9.3 [46]</td>
<td>9.3–9.9 [41]</td>
<td>10.0–10.6 [49]</td>
</tr>
<tr>
<td>IGF-I</td>
<td>143 (132, 153)</td>
<td>140 (129, 151)</td>
<td>147 (136, 158)</td>
</tr>
</tbody>
</table>

1 All nutrients except alcohol were energy-adjusted by using the residual method (26). n in brackets.
2 Regression coefficients (95% CI) from linear regression models adjusted for age.
3 P value for trend based on the continuous measure of the dietary variables.
4 Mean; 95% CI in parentheses (all such values).

<table>
<thead>
<tr>
<th>Quintile of dietary intake</th>
<th>2-SD difference in intake</th>
<th>β (95% CI)</th>
<th>P for β</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Low)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red meat (g/d)</td>
<td>22.6–40.0 [45]</td>
<td>40.1–50.9 [44]</td>
<td>51.0–68.5 [46]</td>
</tr>
<tr>
<td>IGF-I</td>
<td>143 (136, 150) +</td>
<td>156 (141, 171)</td>
<td>145 (132, 158)</td>
</tr>
<tr>
<td>Fish and seafood (g/d)</td>
<td>3.8–16.5 [45]</td>
<td>16.6–23.3 [45]</td>
<td>23.4–39.9 [4]</td>
</tr>
<tr>
<td>IGF-I</td>
<td>156 (142, 170)</td>
<td>129 (116, 143)</td>
<td>146 (133, 160)</td>
</tr>
<tr>
<td>Poultry (g/d)</td>
<td>0 [183]</td>
<td>0 [183]</td>
<td>0 [183]</td>
</tr>
<tr>
<td>Total milk (g/d)</td>
<td>0 [78]</td>
<td>0.1–27.8 [13]</td>
<td>27.9–132.2 [45]</td>
</tr>
</tbody>
</table>

1 2 SDs for each food item were as follows: red meat, 31.2 g/d; fish, 17.0 g/d; poultry, 5.5 g/d; and total milk, 136 g/d. n in brackets.
2 Regression coefficients (95% CI) from linear regression models adjusted for age.
3 P value for trend based on continuous measure of the food item.
4 Mean; 95% CI in parentheses (all such values).
may be due to chance. However, our findings are consistent with those of previous studies. Our results are most directly generalizable to well-nourished middle-aged and elderly men.

Studies in animals and children have shown that restricted intakes of energy, protein, and zinc are associated with lower concentrations of IGF-I (9–11, 27–29). A positive association between zinc intake and IGF-I concentrations was also observed in a study of postmenopausal women (30). Likewise, recent analyses of a subgroup of men from the Health Professionals Follow-Up Study (14) and a subgroup of women from the Nurses’ Health Study (13) indicated a positive association between zinc intake and IGF-I concentrations. Those 2 studies further reported that individuals with a high protein intake had higher IGF-I concentrations than those with a low protein intake.

A positive association between intake of protein and IGF-I has been reported also in other studies (17, 18), although not in all (19, 20). These inconsistencies may reflect different ranges of protein intakes studied, different characteristics of study populations, and validity of measurement of protein intake. In the present study, we reduced random within-person variation by using the average dietary intakes of fourteen 24-h dietary interviews. This should improve validity compared with previous studies in which dietary intakes were assessed only at one occasion with a dietary questionnaire.

Our findings that show a significant positive association between red meat consumption and IGF-I concentrations are consistent with results of some (12, 16), but not all (13–15), previous cross-sectional studies. Furthermore, we observed that men with a high consumption of fish and shellfish had higher IGF-I concentrations than men with low consumption. Although our results for fish did not achieve statistical significance (possibly owing to a relatively small sample size), they are in accord with previous studies in men (14) and women (13).

An increasing body of evidence indicates that a high consumption of red meat may increase the risk of prostate (31) and colorectal cancers (32). In addition, high IGF-I concentrations have been associated with increased risk of these 2 cancers (1), thus raising the possibility that diet may affect cancer risk by modulation of the IGF-system. There is some evidence that IGF-I may predict the risk of advanced-stage prostate cancer only (33) and not of earlier stages (33, 34). In the present study, the concentration of serum IGF-I in men with high consumption of red meat is of the same order of magnitude as reported by other studies in which an association between high serum IGF-I and prostate cancer risk was reported (35).

Several strengths and potential limitations of our study merit comment. The main strengths include its population-based design and the detailed dietary data. The average intake of fourteen 24-h dietary interviews performed over 1 y was used to assess long-term dietary intake. This accurate dietary assessment method would account for dietary changes over time and minimize random error in the measurement of diet. This study was limited by its cross-sectional nature; therefore, we cannot determine the temporal association between changes in dietary intake and IGF-I concentrations. Another limitation is the lack of measurement of free-circulating IGF-I, which may better reflect IGF-I bioactivity than total IGF-I (36). We also did not have data on IGFBP-3, which may regulate IGF-I bioactivity.

In conclusion, in this population-based study, we observed positive associations between intakes of protein, zinc, red meat, and fish and IGF-I concentrations. In view of the potential clinical and public health importance of these findings, the association between dietary intake and IGF-I concentrations in well-nourished free-living adults deserves further study.

SCL, KW, KB, and AW contributed to the study concept and design, interpreted the results, and critically reviewed the manuscript. SCL conducted the statistical analyses and wrote the manuscript. All authors reviewed the final version of the manuscript. None of the authors had any conflicts of financial or personal interest.

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Effects of school milk intervention on cortical bone accretion and indicators relevant to bone metabolism in Chinese girls aged 10–12 y in Beijing1–3

Kun Zhu, Xueqin Du, Chris T Cowell, Heather Greenfield, Barbara Blades, Timothy A Dobbins, Qian Zhang, and David R Fraser

ABSTRACT
Background: We previously reported that increased milk consumption enhances growth and bone mineral accretion in Chinese girls aged 10–12 y.
Objective: Our objective was to evaluate the effects of milk supplementation on cortical bone accretion and to study the physiologic mechanisms underlying the observed changes in bone.
Design: Chinese girls aged 10 y were randomly assigned into calcium-fortified milk (Ca milk), calcium and vitamin D–fortified milk (CaD milk), and control groups according to their schools in a 24-mo school milk intervention trial. Periosteal and medullary diameters of metacarpal bone were measured at baseline and 24 mo in the Ca milk (n = 177), CaD milk (n = 210), and control (n = 219) groups. Insulin-like growth factor I (IGF-I), parathyroid hormone (PTH), bone alkaline phosphatase (BAP), osteocalcin, and deoxypyridinoline concentrations were measured at baseline and at 12 and 24 mo in the Ca milk (n = 43), CaD milk (n = 44), and control (n = 41) groups.
Results: After adjustment for pubertal status and clustering by school, 24-mo supplementation led to greater increases in periosteal diameter (1.2%) and cortical thickness (5.7%) and to smaller gains in medullary diameter (6.7%) than did the control (P < 0.05). The CaD milk group had lower serum BAP at 12 mo (19.9%) and lower serum PTH at 12 (46.2%) and 24 (16.4%) mo than did the control group (P < 0.05). The effect of milk supplementation on increasing IGF-I concentrations at 24 mo (16.7–23.3%) was significant in individual analyses but not after adjustment for clustering by school.

KEY WORDS Fortified milk, Chinese girls, cortical bone accretion, bone alkaline phosphatase, insulin-like growth factor I

INTRODUCTION
The achievement of optimal peak bone mass is important for the prevention of osteoporosis in later life. One of the main focuses of lifestyle modification for peak bone mass is dietary calcium intake. Prospective studies have shown positive effects of dietary supplementation with calcium and dairy products on bone mineral acquisition based on measurements of bone mineral content (BMC) or areal bone mineral density (BMD) in children and adolescents from different countries with background calcium intakes ranging from 280 to 980 mg/d (1–11). The effects of milk or calcium intervention on periosteal and endocortical apposition of cortical bone have not been studied in children and adolescents, even though periosteal apposition and cortical thickness play an important role in building up bone structural strength during growth (12, 13).

Measurement of biochemical markers of bone turnover can provide an estimate of the rate of bone formation and resorption (14). Parathyroid hormone (PTH) is a regulator of mineral homeostasis and bone remodeling (15). Insulin-like growth factor I (IGF-I) promotes longitudinal bone growth and bone formation (16, 17), and high intakes of milk but not of meat were reported to increase serum IGF-I concentrations in children (18). Measurement of these indicators relevant to bone metabolism and development during intervention trials could provide useful information about the physiologic mechanisms by which foods and nutrients affect bone growth. Reduced rates of bone remodeling have been reported to be associated with increased bone mineral accretion in calcium supplementation trials in children (11, 19) but not in the milk supplementation trial with English adolescent girls (9). However, the latter study indicated that some of the anabolic effects of milk on bone may be mediated by increased IGF-I concentrations in supplemented subjects, which may have enhanced periosteal bone apposition and led to a slightly larger total-body bone area.

In a school milk intervention study in Beijing girls aged 10 y at baseline (20), we showed that after 24 mo, the 2 groups that received dietary milk supplement (milk fortified with calcium with or without vitamin D) had increases significantly greater
than the control group in total body size–adjusted BMC (by 1.2–2.4%), total-body BMD (by 3.2–5.3%), and height (by 0.6–0.7%). The group receiving milk fortified with vitamin D also had a significantly improved vitamin D status than did the group receiving milk alone or the control group (plasma 25-hydroxyvitamin D concentrations for vitamin D–fortified milk group: 47.6 ± 23.4 nmol/L; milk alone group, 17.9 ± 9.0 nmol/L; control group, 19.4 ± 10.2 nmol/L; P < 0.0005). The aim of this report is to analyze further the effects of increased milk consumption on periosteal and endocortical apposition of cortical bone through measurements of metacarpal morphometry and to study the physiologic mechanisms for higher growth and bone mineral accretion rate and any effect on cortical bone gain by measurements of indicators relevant to bone metabolism.

SUBJECTS AND METHODS

Subjects and study design

A total of 757 urban Beijing girls aged 10 y participated in a 24-mo school milk intervention trial. The girls were from 9 randomly selected schools in one district in urban Beijing. For administrative reasons, local authority approval was given for only 9 schools to participate in this trial. Subjects were randomly assigned into 3 groups according to their schools, thus ensuring that the schools in the different groups had comparable socioeconomic circumstances. For both ethical and practical reasons it was not possible to randomize within schools so that different milk supplements or no milk would have been provided to different students in the same class. There were 238 girls in the calcium-fortified milk (Ca milk) group who received 330 mL calcium-fortified ultra high temperature (UHT) milk per school day, 260 girls in the calcium and vitamin D–fortified milk (CaD milk) group who received 330 mL calcium and vitamin D–fortified UHT milk per school day, and 259 girls in the control group who received no supplementary milk and consumed their habitual diet during the study period. A subsample of 150 subjects (50 from each group) at the start of the trial and a subsample of 240 subjects (80 from each group) after 12 and 24 mo of dietary intervention were further randomly drawn by a systematic sampling procedure for biochemical measurements. More subjects were selected at 12 and 24 mo because cross-sectional comparisons were also planned for these 2 time points (data not shown). Calculated sample sizes for the intervention trial and the subsequent biochemical analyses were 525 and 43 subjects, respectively, with the power of 90% and 5% risk of a type I error, to detect a difference of 0.02 nmol/L in BMD and a difference of 70 ± 100 nmol/L in plasma IGF-I concentration (21). BMD was used to determine the appropriate sample size, as more published data for this variable were available at the time the study was designed. However, considering the evidence of previously published supplementation trials with children and adolescents which have shown supplementation effects with samples sizes of 24–130 in each group subject to enough dosage (>300 mg Ca/d) and duration (>18 mo) (1–6), the sample size for the trial was determined as 250 for each group, as a compromise between the calculated sample size of 525 and the actual sample sizes (24–130) for the studies cited. The sample size for the biochemical analyses was determined as 50 in each group, assuming a 10% dropout rate.

The UHT milk for this project was specially formulated by Murray Goulburn Co-operative Co Ltd (Brunswick, Australia) to comply with both Chinese and Australian food regulations. The milk was fortified with a milk calcium salt (NatraCal) to give a total calcium content of 560 mg (the equivalent of the calcium in 500 mL regular milk) within a volume of 330 mL. This is to keep the supplementation milk within the amount readily consumed on each occasion and to avoid any problems of lactose intolerance associated with milk overload. Each carton of milk (330 mL) also contained 10 g fat and 10 g protein; for milk used in the CaD milk group, 5–8 µg vitamin D was added. After correcting for weekends and holidays, when no intervention milk was consumed, the average daily supplementation over the 24 mo was 144 mL milk, containing 245 mg Ca and, for the CaD milk group, 3.33 µg vitamin D. A detailed description of the composition of the milk supplements and the added milk calcium salts has been given elsewhere (20). The study was carried out with the approval of the Ethics Committees of the University of Sydney, Australia, and the Institute of Nutrition and Food Hygiene of the Chinese Academy of Preventive Medicine (now the Chinese Center for Disease Control and Prevention). A consent form in Chinese was signed by the parents of all the study participants.

Metacarpal morphometry and bone age

Posteroanterior X-ray radiographs of the nondominant hand and wrist were taken at baseline and 24 mo with the use of a Toshiba KXO-152 X-ray apparatus (Tokyo) at an average setting of 5 mA and 46 kV. The film focus distance was 110 cm, and the central beam was focused on the wrist. The radiation dose was 0.01 mSv per exposure. Complete X-ray radiographs at baseline and 24 mo were obtained from 606 girls (Ca milk group, n = 177; CaD milk group, n = 210; and control group, n = 219). Periosteal diameter (outer width) and medullary diameter (inner width) of the midpoint of the second metacarpal and the length of the second metacarpal were measured by one examiner with a digital caliper (Mitutoyo, Kawasaki, Japan). The intraobserver reliability was assessed by repeating the measurements of 28 hand radiographs at different times over 1 mo. The CVs were <1% for outer width, <2% for inner width, and <1% for length. The combined cortical thickness (CCT) was calculated as periosteal diameter – medullary diameter. Bone age (to the nearest 0.1 y) was determined from these radiographs by assessing the development stages of metacarpals, phalanges, and carpal according to the Chinese standard (22).

Biochemical analysis

Samples of overnight fasting blood (drawn between 0630 and 0900) and urine (second morning void, 0730–0900) were obtained at baseline and at 12 and 24 mo in the subsample. For each subject, repeat sample collection was at the same time of day, to minimize the effects of diurnal variation. Blood samples were collected in untreated tubes for serum and in lithium-heparin tubes for plasma. Urine samples were collected in light-protected containers at room temperature without preservatives. An aliquot of each sample was frozen within 4 h after the end of the collection period and stored at −20 °C. Frozen samples were transferred in dry ice (−78.5 °C) by air from Beijing to Sydney on each occasion and then stored at −70 °C until analysis. Urine samples were protected from light during assay as required by assay instructions. Serum and plasma samples were handled as for other routine analytic procedures.
Serum bone alkaline phosphatase (BAP) was assayed by single antibody immunoassay (Alkphase-B; Metra Biosystems Inc, Mountain View, CA). The intraassay CV was 5.0% and the interassay CV was 5.2% at 72 U/L. Plasma osteocalcin was evaluated by a two-site enzyme-linked immunosorbent assay (N-MID Osteocalcin; Osteometer BioTech, Copenhagen). The intraassay CV was 5.4% and the interassay CV was 6.5% at 17 ng/mL. Urinary free deoxypyridinoline was measured by competitive immunoassay (Immulite Pyrilinks-D; Diagnostic Products Corporation, Los Angeles). The intraassay CV was 4.0% and the interassay CV was 4.7% at 97 nmol/L. To correct for variations in urinary flow, deoxypyridinoline results were normalized to the urinary creatinine concentration and expressed as a ratio to urinary creatinine excretion (in mmol/mmol). Urinary creatinine was measured by Beckman Clinical Systems (Synchron CX5; Beckman Coulter Inc, Fullerton, CA) by using an enzymatic method. Serum PTH was quantified by immunometric assay (Immulite Intact PTH; Diagnostic Products Corporation, Los Angeles). The intraassay CV was 5.4% and the interassay CV was 5.0% at 86 pg/mL. Plasma IGF-I was measured by double antibody radioimmunoassay (Bioclone, Marrickville, NSW, Australia). This method includes a simple extraction step with acid-ethanol in which IGF-I was separated from its binding protein in plasma. The intraassay CV was 3.4% and the interassay CV was 4.3% at 263 ng/mL.

Other measurements

Health history of the subjects and family members and family socioeconomic status were obtained by a general information questionnaire at baseline. The following assessments were made at baseline and at 12 and 24 mo: body weight by an electronic scale (Thinner, Fairfield, WI), height and sitting height by body and sitting height measures (TG-III, Beijing, China), BMC, bone area, and BMD of total body and forearm by dual-energy X-ray absorptiometry (XR-36; Norland Medical Systems Inc, Fort Atkinson, WI), dietary intakes by 7-d unweighed food record (24-h recall diary for 7 d) at baseline and 3-d food record at 12 and 24 mo (number of days were reduced at 12 and 24 mo because of subject fatigue), and pubertal stage of breast and pubic hair development according to Tanner’s definitions of the 5 stages of puberty (23). Date of menarche was recorded.

Statistical analysis

Descriptive statistics are reported as means ± SDs, and differences as mean (95% CI) for all variables, unless otherwise indicated. Baseline values among the 3 groups were compared by using one-factor analysis of variance (ANOVA) and chi-square test when appropriate. A two-factor repeated-measures ANOVA was used to test time × supplementation interactions. Post hoc analysis was carried out with Tukey’s honestly significant difference test. In case of significant time × supplementation interaction, the within-group comparison of values at 12 and 24 mo with baseline was carried out by one-factor repeated-measures ANOVA with a Bonferroni-corrected $P$ value of 0.025 for measurements made at 3 time points.

To allow for clustering by school, adjusted analyses were conducted by using the linear mixed model, with school defined as a random effect (24). As outcome variables displayed a skewed distribution, they were transformed by using a log transformation. Estimates of the strength of clustering within school are provided by the intracluster correlation coefficient. Outcomes were analyzed by adjusting for the baseline value, intervention group, and potential confounding variables such as Tanner stage, menarchal status, and bone age. As the adjusted analyses are based on the natural log-transformed outcome variables, the differences between each of the intervention groups and the control group were calculated as percentage differences. For metacarpal morphometry measurements, when no significant difference between the 2 interventions was observed, intervention groups were pooled and compared with the control group to assess the combined effect of the interventions. The supplementation effects independent of potential confounding factors were also analyzed at the individual level, with a multiple regression model with backward elimination (11, 25, 26). The significance level for test statistics was set at $P < 0.05$. All data were analyzed with SPSS (version 10.0; SPSS Inc, Chicago) and SAS (SAS for WINDOWS version 8.2; SAS Institute Inc, Chicago).

RESULTS

Six hundred ninety-eight (92.2%) of the original 757 subjects completed the trial. Overall compliance among subjects who completed the study was close to 100% according to school milk consumption record, and no subject withdrew from the study because of adverse effects from milk consumption. Data for 606 girls with complete hand X-ray radiographs and 128 girls with complete biochemical analysis data are presented in this paper. No significant differences were observed in baseline measured characteristics (anthropometry, dietary intakes, pubertal development, and bone mineral measurements) between girls who left the study and girls who completed it and between girls whose hand X-ray or biochemical analysis data are not available and girls for whom these data are available.

The characteristics of subjects at baseline and after 24 mo of the trial are shown in Table 1. At baseline, except for the number of years of education of the parents, no significant differences were observed among the 3 groups in any of the variables listed. At 24 mo, subjects in all 3 groups were significantly heavier and taller than at baseline (Table 1) [effects of supplementation on height and weight have been discussed elsewhere (20)]. Both supplemented groups had significantly higher calcium intakes at 24 mo than their baseline calcium intakes and the calcium intake of the control group at 24 mo (Table 1). No significant differences were observed among the 3 groups in protein intake and percentage of subjects at each Tanner stage (breast and pubic hair) at 24 mo, but a trend was observed for a higher proportion of girls in the Ca milk and CaD milk groups to have passed through menarche at 24 mo ($P = 0.065$).

No significant differences were observed among the 3 groups at baseline in any of the metacarpal morphometry measurements. All 3 groups of girls experienced increases in periosteal diameter, CCT, and length of the second metacarpal, whereas medullary diameter decreased in the Ca milk group, increased in the control group, and remained unchanged in the CaD milk group over the 24-mo period (Table 2). After 24 mo, in comparison with the control intervention, supplementation had led to significantly greater increases in periosteal diameter, CCT, and length of the second metacarpal and to smaller gains in medullary diameter after adjustment for baseline value, bone age, Tanner breast and pubic hair development stage, and menarchal status at 24 mo.
and clustering by school (Table 3). Percentage changes in periosteal diameter were positively correlated with percentage changes in total-body BMC ($r = 0.418, P < 0.001$), bone area ($r = 0.233, P < 0.001$), and BMD ($r = 0.139, P = 0.015$). Percentage changes in medullary diameter were negatively correlated with those of total-body BMC ($r = -0.163, P = 0.004$) and BMD ($r = -0.234, P < 0.001$). Percentage changes in CCT were positively correlated with those of total-body BMC ($r = 0.329, P < 0.001$) and BMD ($r = 0.251, P < 0.001$).

Concentrations of serum BAP, serum PTH, plasma osteocalcin, plasma IGF-I, and urinary deoxypyridinoline were not significantly different among the 3 groups at baseline (Table 2). Over the 24 mo, all 3 groups experienced increases in deoxypyridinoline concentrations, whereas osteocalcin concentration remained unchanged. The control group experienced increases in serum BAP concentrations. Concentrations of plasma IGF-I and serum PTH at 24 mo were significantly higher than those at baseline in all 3 groups. The CaD milk group also had concentrations significantly higher for IGF-I and significantly lower for PTH at 12 mo than at baseline (Table 2). Comparisons between supplemented and control groups were made for variables (BAP, IGF-I, and PTH) with significant interaction of time X supplementation (Table 4). After adjustment for the baseline value, Tanner breast and pubic hair development stage, menarchal status, and clustering by school, the CaD milk group had concentrations significantly lower for serum BAP (19.9%) at 12 mo and concentrations significantly lower for serum PTH at 12 mo (46.2%) and 24 mo (16.4%) than did the control group. The effects of milk supplementation on reducing serum BAP concentrations at 12 mo in the Ca milk group and on increasing IGF-I concentrations at 24 mo in both supplemented groups were significant at the level of individual analysis but not after adjustment for clustering by school.

**DISCUSSION**

This study showed that in Chinese girls aged 10 y at the start of the trial, 24-mo milk supplementation (fortified with calcium with or without vitamin D) was associated with a greater increase in both periosteal and endocortical apposition than in unsupplemented control subjects. These changes led to greater gain in cortical thickness in supplemented subjects.

Before puberty, periosteal apposition increases bone width in both boys and girls. During puberty, most of the increase in cortical thickness is achieved by periosteal apposition in boys and by medullary contraction in girls in the upper limbs (27, 28). From age 10 to 12 y, when most of our subjects experienced early

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### Table 1

Characteristics of the supplemented and control groups at baseline and 24 mo

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>24 mo</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Ca milk group ($n = 177$)</td>
<td>CaD milk group ($n = 210$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>10.1 ± 0.4$^2$</td>
<td>10.1 ± 0.3</td>
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<tr>
<td>Bone age (y)$^6$</td>
<td>9.8 ± 1.0</td>
<td>10.0 ± 1.1</td>
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<tr>
<td>Height (cm)$^6$</td>
<td>140.2 ± 6.1</td>
<td>141.3 ± 6.9</td>
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<tr>
<td>Weight (kg)$^6$</td>
<td>33.8 ± 7.2</td>
<td>33.6 ± 7.0</td>
</tr>
<tr>
<td>Calcium intake (mg/d)$^7$</td>
<td>421.7 ± 146.1</td>
<td>422.2 ± 164.6</td>
</tr>
<tr>
<td>Protein intake (g/d)$^7$</td>
<td>52.0 ± 14.3</td>
<td>53.7 ± 15.0</td>
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Subjects at Tanner breast stage (%)

<table>
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<th>CaD milk group</th>
<th>Control group</th>
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</thead>
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<tr>
<td>1</td>
<td>42.3</td>
<td>39.2</td>
<td>44.7</td>
</tr>
<tr>
<td>2</td>
<td>50.3</td>
<td>50.2</td>
<td>50.2</td>
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<tr>
<td>3</td>
<td>7.4</td>
<td>10.5</td>
<td>5.0</td>
</tr>
<tr>
<td>4–5</td>
<td>0</td>
<td>0</td>
<td>13.6</td>
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Subjects at Tanner pubic hair stage (%)

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<th>Control group</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>95.4</td>
<td>95.2</td>
<td>96.8</td>
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<tr>
<td>2</td>
<td>4.6</td>
<td>4.8</td>
<td>3.2</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>26.1</td>
</tr>
<tr>
<td>4–5</td>
<td>0</td>
<td>0</td>
<td>7.4</td>
</tr>
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</table>

Postmenarchal (%)

<table>
<thead>
<tr>
<th>CaD milk group</th>
<th>CaD milk group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7</td>
<td>1.9</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Monthly family income (RMB)

<table>
<thead>
<tr>
<th>CaD milk group</th>
<th>CaD milk group</th>
<th>Control group</th>
</tr>
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<tbody>
<tr>
<td>2237 ± 1054</td>
<td>2314 ± 1235</td>
<td>2108 ± 1120</td>
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</table>

Monthly food expenditure (RMB)

<table>
<thead>
<tr>
<th>CaD milk group</th>
<th>CaD milk group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1054 ± 426</td>
<td>1024 ± 440</td>
<td>973 ± 391</td>
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</table>

Education of parents (y)

<table>
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<tr>
<th>CaD milk group</th>
<th>CaD milk group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.9 ± 1.9$^7$</td>
<td>14.0 ± 1.8$^8$</td>
<td>13.3 ± 1.9</td>
</tr>
</tbody>
</table>

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$^1$ Ca milk group, received 330 mL Ca-fortified milk on school days; CaD milk group, received 330 mL Ca- and vitamin D-fortified milk on school days.  
$^2$ ± SD (all such values).  
$^3$ Significant interaction between group and time, $P < 0.001$ (two-factor repeated-measures ANOVA with interaction).  
$^4$ Significantly different from baseline, $P < 0.001$ (one-factor repeated-measures ANOVA).  
$^5$ Significantly different from the control group at 24 mo, $P < 0.001$ (two-factor repeated-measures ANOVA, post hoc Tukey’s test).  
$^6$ Significant main effect of time, $P = 0.004$ (two-factor repeated-measures ANOVA with interaction).  
$^7$ $^8$ Significantly different from the control group at baseline (one-factor ANOVA, post hoc Tukey’s test): $^7P = 0.005$, $^8P < 0.001$.  

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Supplementation of dairy products or calcium in children and adolescents has shown positive effects of supplementation on bone mineral accretion according to BMC or areal BMD measurements (1–11). For bone sites where cortical bone is predominant, a greater BMC or areal BMD could be caused by a greater cortical thickness (from greater periosteal apposition, greater endocortical apposition, or both) or a greater volume BMD.

However, the changes at the bone surfaces during dietary supplementation with calcium and dairy products have, to our knowledge, not previously been studied in children and adolescents. In a 3-y calcium supplementation study in perimenopausal women, it was found that decrease in metacarpal cortical thickness was 1% lower in the supplemented group than in the control group (29). The results of the present study showed that increases in periosteal diameter and CCT and reduction in medullary diameter are associated with increases in total-body BMC and BMD, indicating that the greater increase in total-body BMC and BMD of the supplemented groups could be explained partly by enhanced periosteal and endocortical bone apposition.

Although the increases in total-body BMC and size-adjusted BMC were 2.0% and 1.3% greater, respectively, in the CaD milk group than the Ca milk group (20), we found that there were no significant differences between the 3 groups for any variable at baseline (one-factor ANOVA, post hoc Tukey’s test).

The first and second numbers reflect the number of subjects who underwent the metacarpal morphometry measurements and the bone metabolism measurements, respectively.

Significant main effects of time, \( P < 0.05 \) (two-factor repeated-measures ANOVA with interaction).

Significantly different from baseline (one-factor repeated-measures ANOVA with Bonferroni correction): \( \times P < 0.001, \quad 3 P < 0.002, \quad 6 P = 0.013, \quad 4 P = 0.001, \quad 5 P = 0.007 \).

Significant main effects of time, \( P < 0.001 \) (two-factor repeated-measures ANOVA with interaction).

Ca milk group \((n = 177, 43)\)

<table>
<thead>
<tr>
<th>Measure</th>
<th>Ca milk group</th>
<th>CaD milk group</th>
<th>Control group</th>
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<tr>
<td>Baseline</td>
<td>6.45 ± 0.53</td>
<td>6.38 ± 0.57</td>
<td>6.41 ± 0.56</td>
</tr>
<tr>
<td>24 mo</td>
<td>7.02 ± 0.57†</td>
<td>6.97 ± 0.57†</td>
<td>6.93 ± 0.61†</td>
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<tr>
<td>Medullary diameter (mm)</td>
<td>3.09 ± 0.58†</td>
<td>3.03 ± 0.65†</td>
<td>2.95 ± 0.63†</td>
</tr>
<tr>
<td>Baseline</td>
<td>2.99 ± 0.70†</td>
<td>3.00 ± 0.74†</td>
<td>3.07 ± 0.70†</td>
</tr>
<tr>
<td>CCT (mm)</td>
<td>3.36 ± 0.49†</td>
<td>3.35 ± 0.56†</td>
<td>3.46 ± 0.51†</td>
</tr>
<tr>
<td>Baseline</td>
<td>4.03 ± 0.62†</td>
<td>3.97 ± 0.62†</td>
<td>3.87 ± 0.57†</td>
</tr>
<tr>
<td>Length of 2nd metacarpal (mm)</td>
<td>53.85 ± 2.99</td>
<td>54.20 ± 3.76</td>
<td>54.18 ± 3.48</td>
</tr>
<tr>
<td>Baseline</td>
<td>60.08 ± 3.19†</td>
<td>60.17 ± 3.63†</td>
<td>59.88 ± 3.47†</td>
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<tr>
<td>BAP (U/L)</td>
<td>157.39 ± 45.10</td>
<td>151.38 ± 45.54</td>
<td>148.90 ± 40.34</td>
</tr>
<tr>
<td>Baseline</td>
<td>12 mo</td>
<td>137.53 ± 37.67</td>
<td>179.38 ± 62.94†</td>
</tr>
<tr>
<td>24 mo</td>
<td>167.11 ± 54.49</td>
<td>159.10 ± 63.83</td>
<td>177.91 ± 75.13º</td>
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<tr>
<td>OC (ng/mL)</td>
<td>108.07 ± 37.01</td>
<td>109.52 ± 35.82</td>
<td>121.00 ± 42.49</td>
</tr>
<tr>
<td>Baseline</td>
<td>12 mo</td>
<td>108.45 ± 52.46</td>
<td>132.54 ± 53.05</td>
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<tr>
<td>24 mo</td>
<td>107.02 ± 38.75</td>
<td>101.71 ± 42.98</td>
<td>115.76 ± 49.74</td>
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<tr>
<td>Dpd (nmol/mmol)</td>
<td>19.40 ± 6.13</td>
<td>21.67 ± 6.68</td>
<td>21.60 ± 6.02</td>
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<tr>
<td>Baseline</td>
<td>12 mo</td>
<td>33.08 ± 9.94</td>
<td>29.79 ± 9.72</td>
</tr>
<tr>
<td>24 mo</td>
<td>30.10 ± 8.39</td>
<td>27.93 ± 8.55</td>
<td>30.18 ± 10.55</td>
</tr>
<tr>
<td>IGF-I (ng/mL)</td>
<td>245.97 ± 85.77</td>
<td>258.56 ± 91.64</td>
<td>260.71 ± 88.15</td>
</tr>
<tr>
<td>Baseline</td>
<td>12 mo</td>
<td>294.89 ± 100.21</td>
<td>264.63 ± 93.94</td>
</tr>
<tr>
<td>24 mo</td>
<td>413.10 ± 126.54⁴</td>
<td>442.21 ± 131.58⁴</td>
<td>355.30 ± 120.80⁹</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td>36.85 ± 21.26</td>
<td>37.09 ± 24.52</td>
<td>40.18 ± 20.11</td>
</tr>
<tr>
<td>Baseline</td>
<td>12 mo</td>
<td>22.58 ± 10.85⁴</td>
<td>46.12 ± 33.98</td>
</tr>
<tr>
<td>24 mo</td>
<td>63.35 ± 28.79⁴</td>
<td>50.92 ± 26.45¹⁰</td>
<td>72.17 ± 45.89⁴</td>
</tr>
</tbody>
</table>

1 All values are \( \bar{x} \pm SD \). Ca milk group, received 330 mL Ca-fortified milk on school days; CaD milk group, received 330 mL Ca- and vitamin D–fortified milk on school days; CCT, combined cortical thickness; BAP, bone alkaline phosphatase; OC, osteocalcin; Dpd, deoxypyridinoline; IGF-I, insulin-like growth factor I; PTH, parathyroid hormone. There were no significant differences between the 3 groups for any variable at baseline (one-factor ANOVA, post hoc Tukey’s test).

2 The first and second numbers reflect the number of subjects who underwent the metacarpal morphometry measurements and the bone metabolism measurements, respectively.

3 Significant interactions between group and time, \( P < 0.05 \) (two-factor repeated-measures ANOVA with interaction).

4–6\,8–10 Significantly different from baseline (one-factor repeated-measures ANOVA with Bonferroni correction); \( 4 P < 0.001, \quad 5 P = 0.002, \quad 6 P = 0.013, \quad 8 P = 0.001, \quad 9 P = 0.007 \).

7 Significant main effects of time, \( P < 0.001 \) (two-factor repeated-measures ANOVA with interaction).

8 \( n = 33 \) and 20 at 12 mo for the Ca milk and CaD milk groups, respectively, because of assay problems.
and 219 (control group). For analysis at the cluster level, school (BAP, bone alkaline phosphatase; IGF-I, insulin-like growth factor I; PTH, parathyroid hormone; ICC, intracluster correlation coefficient. For analysis at the individual level, CCT, combined cortical thickness; ICC, intracluster correlation coefficient. For analysis at the individual level with a multiple regression model, adjusted for baseline value, Tanner stage, and menarcheal status at 12 and 24 mo (see Subjects and Methods for details).

## TABLE 3

<table>
<thead>
<tr>
<th></th>
<th>Adjusted percentage difference in outcome at 24 mo relative to control group(^1)</th>
<th>Adjusted percentage difference in outcome at 24 mo relative to control group, allowing for clustering by school(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Periosteal diameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca milk group</td>
<td>0.8 (0.2, 1.4)</td>
<td>0.004</td>
</tr>
<tr>
<td>CaD milk group</td>
<td>1.3 (0.7, 1.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pooled interventions</td>
<td>1.0 (0.6, 1.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Medullary diameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca milk group</td>
<td>−7.4 (−10.1, −4.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CaD milk group</td>
<td>−5.2 (−7.8, −2.6)</td>
<td>&lt;0.001</td>
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<tr>
<td>Pooled interventions</td>
<td>−6.3 (−7.5, −5.1)</td>
<td>&lt;0.001</td>
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<tr>
<td>CCT</td>
<td></td>
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<tr>
<td>Ca milk group</td>
<td>5.4 (3.2, 7.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CaD milk group</td>
<td>4.5 (2.5, 6.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pooled interventions</td>
<td>4.9 (3.1, 6.7)</td>
<td>&lt;0.001</td>
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<tr>
<td>Length of 2nd metacarpal</td>
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<tr>
<td>Ca milk group</td>
<td>0.9 (0.5, 1.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CaD milk group</td>
<td>1.0 (0.6, 1.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pooled interventions</td>
<td>0.9 (0.5, 1.3)</td>
<td>&lt;0.001</td>
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\(^1\) Ca milk group, received 330 mL Ca-fortified milk on school days; CaD milk group, received 330 mL Ca- and vitamin D–fortified milk on school days; CCT, combined cortical thickness; ICC, intracluster correlation coefficient. For analysis at the individual level, n = 177 (Ca milk group), 210 (CaD milk group), and 219 (control group). For analysis at the cluster level, n = 3 per group.

\(^2\) Analysis at the individual level with a multiple regression model, adjusted for baseline value, bone age, Tanner stage, and menarcheal status at 24 mo (see Subjects and Methods for details).

\(^3\) Analysis at the cluster level with a linear mixed model, adjusted for baseline value, bone age, Tanner stage, menarcheal status at 24 mo, and clustering by school (see Subjects and Methods for details).

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differences between these 2 groups in increases in periosteal diameter and CCT. Therefore, we suggest that the greater increases in BMD and size-adjusted BMC of the CaD milk group could be the consequence of a greater increase in volume BMD in this group. This finding is a further indication of the role of vitamin D in promoting the supply of calcium from the diet for bone mineralization.

IGF-I promotes longitudinal bone growth and cortical and trabecular bone formation (16, 17, 30). English adolescent girls who received milk supplementation were reported to have higher concentrations of IGF-I (9). In a study in 8-y-old boys, high intakes of milk but not of meat were reported to increase serum IGF-I concentrations (18). We suggest that the observed greater gain in height (20), in length of the second metacarpal, and in

## TABLE 4

<table>
<thead>
<tr>
<th></th>
<th>Adjusted percentage difference in outcome at 12 and 24 mo relative to control group(^2)</th>
<th>Adjusted percentage difference in outcome at 12 and 24 mo relative to control group, allowing for clustering by school(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 mo</td>
<td>24 mo</td>
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<tr>
<td></td>
<td>Mean (95% CI)</td>
<td>P</td>
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<tr>
<td>BAP</td>
<td></td>
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</tr>
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<td>Ca milk group</td>
<td>−13.6 (−24.2, −3.0)</td>
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<td>CaD milk group</td>
<td>−25.3 (−35.5, −15.1)</td>
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<td>IGF-I</td>
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<td>Ca milk group</td>
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<tr>
<td>CaD milk group</td>
<td>13.0 (−0.1, 26.1)</td>
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<td>PTH</td>
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<tr>
<td>CaD milk group</td>
<td>−63.2 (−89.9, −36.5)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\(^1\) Ca milk group, received 330 mL Ca-fortified milk on school days; CaD milk group, received 330 mL Ca- and vitamin D–fortified milk on school days; BAP, bone alkaline phosphatase; IGF-I, insulin-like growth factor I; PTH, parathyroid hormone; ICC, intracluster correlation coefficient. For analysis at the individual level, n = 43 (Ca milk group), 44 (CaD milk group), and 41 (control group). For analysis at the cluster level, n = 3 per group.

\(^2\) Analysis at the individual level with a multiple regression model, adjusted for baseline value, Tanner stage, and menarcheal status at 12 and 24 mo (see Subjects and Methods for details).

\(^3\) Analysis at the cluster level with a linear mixed model, adjusted for baseline value, Tanner stage, menarcheal status at 12 and 24 mo, and clustering by school (see Subjects and Methods for details).
increased periosteal and endocortical apposition in the supplemented groups in the present study could have resulted from increased IGF-I concentrations in response to the increased milk intake. However, the effect of milk supplementation on increasing IGF-I concentrations at 24 mo was significant only at the level of individual analysis, and the significant difference was lost after adjustment for clustering by school because of a high intraclass correlation (0.25). It is not clear why IGF-I had a high variation between schools. Because samples from the different schools were all assayed together and each assay was well controlled, assay variability is unlikely to be a cause.

Lower serum BAP concentrations were observed in the CaD milk group at 12 mo, associated with reduced PTH secretion after the increased calcium intake and improved vitamin D status. Lower serum BAP concentrations were also indicated in the Ca milk group at 12 mo at the level of individual analysis. Markers of bone turnover during growth reflect both bone modeling and remodeling (19). The present study showed that the supplementation had led to increased bone formation, as reflected by greater periosteal and endocortical apposition. Therefore, the lower BAP concentration in the supplemented groups was unlikely to have resulted from a reduced rate of bone modeling but rather from a reduced rate of bone remodeling. However, no difference was observed among the 3 groups in the other 2 biomarkers of bone turnover measured in this study, osteocalcin and deoxypyridinoline. This absence of any effect may be explained by the observation that these 2 biomarkers are related to longitudinal growth during puberty (31, 32), and a decrease in osteocalcin and deoxypyridinoline concentration resulted from the reduced rate of bone remodeling being offset by an increase in their concentrations associated with the higher growth rate in supplemented groups. The reduced rate of bone remodeling, reflected by reduced BAP concentration in supplemented groups, indicated that the phenomenon known as the “bone remodeling transient” partly mediated the effect of supplementation on bone mineral status in the present study, especially in the CaD milk group (33). Decreased rates of bone turnover were also observed in other calcium supplementation trials in children (1, 11, 19) but not in a milk supplementation trial in adolescent English girls (9). These findings suggest that the reduced rate of bone turnover and reduced PTH concentration in the CaD milk group found in the present study possibly resulted from the fortification of the milk with milk calcium salts and vitamin D.

Although our study was a randomized controlled trial and could, therefore, allow conclusions of causality to be drawn, it does have inherent limitations. Because of practical and ethical considerations and other physical constraints, only 9 schools participated in the study, and subjects were randomly assigned according to their schools. When the analysis was adjusted for the cluster design, some of the significant effects found in the randomized group analysis, including the effects of IGF-1 in both supplemented groups and BAP in the Ca milk group, became obscured because of the reduced effective sample size and power. This limited the interpretation of such findings, although they are likely to indicate the underlying biologic mechanisms. The assessment of cortical bone accretion was based on the traditional method, measurement of metacarpal morphometry. It would be ideal if the changes in periosteal and medullary diameters, cortical thickness, and bone cross-sectional area of sites of interests (such as radius) had been monitored by more advanced techniques, such as peripheral quantitative computed tomography. The second morning void urine sample was used for administrative reasons, although it is preferable to collect total 24-h urine because circadian variation in urinary deoxypyridinoline excretion has been observed (34). Estrogen concentrations were not examined in the study subjects.

Our data showed a positive effect of milk supplementation on periosteal apposition and cortical bone accretion in Chinese girls aged 10–12 y. Such effects, if maintained, could confer greater bone strength in supplemented girls. The positive effects of 24-mo of milk supplementation (fortified with calcium with or without vitamin D) on bone mineral accretion are partly mediated by greater periosteal and endocortical apposition of cortical bone and partly by reduced bone remodeling. Follow-up studies of calcium supplementation trials in children yielded inconsistent results (19, 35–38). It seems that the effects on modeling bone size may persist (37), whereas the effects on remodeling would not (19). A follow-up study, with the same primary school subjects now dispersed to >30 secondary schools, is currently under way to determine whether the effects of short-term supplementation with fortified milk are maintained after the experimental dietary intervention.

We thank the staff of Beijing Xicheng Student Health Institute and the Department of School Nutrition, Institute of Nutrition and Food Safety, for their help with the field work; C Cai, J Liu, and Z Wen from Beijing No. 304 Hospital for hand X-rays and bone densitometry; J Lee from the Children’s Hospital at Westmead for technical support with the biochemical analysis; and J Simpson of the School of Public Health, University of Sydney, for advice on the mixed-model analysis. We appreciate the support from all school principals and teachers involved and the cooperation from all participating students and their parents.

KZ was involved in the conception and design of the study, data collection, and data analysis and drafted the manuscript. XD was involved in the conception and design of the study and in the data collection. CTC was involved in the laboratory analysis consultation and interpretation of the study. HG and DRF were involved in the conception, design, and interpretation of the study. BB was involved in the laboratory analysis consultation. TD was involved in the data analysis with the linear mixed model, which allowed clustering by school. ZQ was involved in the data collection. All authors contributed to the writing of the manuscript. None of the authors had any financial or personal conflicts of interest with any sponsors of this research.

REFERENCES


Quercetin, fruit consumption, and bone mineral density

Dear Sir:

McGartland et al (1) reported a correlation between a diet high in fruit and bone mineral density. Their article includes a discussion of how a low pH stimulates osteoclasts and how fruit’s alkaline-forming properties influence the body’s acid-base balance.

Another mechanism exists that could work in parallel or in synergy with the one proposed by McGartland et al. Diets high in fruit contain high amounts of flavonoids (2). The flavonoid quercetin decreases the differentiation of osteoclast progenitor cells and inhibits the activity of mature osteoclasts (3–5). Quercetin might act together with the alkaline-forming properties of fruit to inhibit osteoclasts and enhance bone mineral density.

The author had no conflicts of interest to report.

Celia M Ross
36 Ridgewood Circle
Wilmington, DE 19809
E-mail: celiamaryross@aol.com

REFERENCES

Reply to CM Ross

Dear Sir:

In her response to our article “Fruit and vegetable consumption and bone mineral density: the Northern Ireland Young Hearts Project,” Ross raises an interesting issue for discussion. We agree that the flavonoid quercetin may inhibit osteoclasts and enhance bone mineral density.

Phytoestrogens comprise a variety of structurally diverse chemicals, with flavonoids as their largest group (1). In our discussion, we stated that phytoestrogens have been identified as being potentially important for bone health (2). Because we did not measure quercetin concentrations in our subjects, we did not specifically mention quercetin in our discussion. We thank Ross for her interest in our article and for highlighting the potential role of quercetin in bone health.

The authors had no conflicts of interest to report.

Claire P McGartland
Paula J Robson
Northern Ireland Center for Food and Health
University of Ulster
Coleraine
United Kingdom
E-mail: c.mcgartland@qub.ac.uk

Liam J Murray
Gordon W Cran
J Maurice Savage
David C Watkins
Department of Epidemiology and Public Health
Queen’s University
Belfast
United Kingdom

Madeleine M Rooney
Rheumatology Department
Musgrave Park Hospital
Belfast
United Kingdom

Colin A Boreham
School of Applied Medical Sciences and Sports Studies
University of Ulster
Jordanstown
United Kingdom

REFERENCES
Dear Sir:

We read with great interest the excellent article by Specker (1), who carried out a comprehensive review of studies that investigated maternal and neonatal outcomes of vitamin D deficiency or supplementation during pregnancy. We would like to add to this discussion an important topic that has become evident recently and that was not addressed by the clinical studies that Specker reviewed: the fact that extrarenal synthesis of 1,25-dihydroxyvitamin D \([1,25(\text{OH})_2D_3] \), or calcitriol, is of great importance for homeostasis in a multitude of tissues, including the immune system, and that this function of vitamin D is likely responsible for the numerous epidemiologic observations that persons who live at higher latitudes, who are more prone to vitamin D deficiency, are at increased risk of not only developing prostate, colon, breast, and other solid tumors; hypertension; and cardiovascular heart disease but also of developing autoimmune diseases, including multiple sclerosis and type 1 diabetes (2–5). Consequently, increasing evidence now indicates that vitamin D deficiency during pregnancy may represent for the fetus a predisposing factor for the future development of a broad variety of diseases, including diseases of the immune system, such as atopic dermatitis or autoimmune diseases (4, 6, 7).

During the past few years, important new immunomodulatory effects of vitamin D analogues have been characterized (4, 7–9). Today, the local synthesis of calcitriol in immune cells is considered to be of great importance for the regulation and control of immune responses. 1,25(\text{OH})_2D_3 inhibits activation of T cells and induces the generation of CD25^+CD4^+ regulatory T cells (4, 7, 9). In dendritic cells, 1,25(\text{OH})_2D_3 inhibits maturation and induces a phenotype that promotes tolerance and inhibits immunity after stimulation with antigen (8, 9). In dendritic cells, calcitriol suppresses expression of major histocompatibility complex II molecules and of costimulatory molecules, including CD40, CD80, and CD86 (8, 9). In these cells, production of interleukin (IL) 10 is stimulated and production of IL-12 inhibited, which leads to suppression of T cell activation. At present, a connection between vitamin D and pathogenesis of atopic dermatitis is discussed. Epidemiologic studies have shown that patients with atopic dermatitis have a lower vitamin D intake than do control subjects (6). Vitamin D analogues suppress in vitro immunoglobulin E production and immunoglobulin E-mediated cutaneous reactions (10, 11).

In conclusion, a growing body of evidence now clearly indicates that adequate vitamin D concentrations during pregnancy are not only necessary to ensure appropriate maternal responses to the calcium demands of the fetus and neonatal handling of calcium, but also of great importance to guarantee the healthy development of a broad variety of tissues, including the immune system. Consequently, vitamin D deficiency during pregnancy may represent for the fetus a predisposing factor for the future development of a multitude of diseases not related to fetal growth and bone metabolism, including diseases of the immune system, such as atopic dermatitis, type 1 diabetes, and other autoimmune diseases.

The authors had no conflicts of interest to report.

Jörg Reichrath
Kerstin Querings

Reply to J Reichrath and K Querings

Dear Sir:

I appreciate the interest of Reichrath and Querings in my review of vitamin D requirements during pregnancy. Those authors noted that there is increasing evidence that vitamin D deficiency during pregnancy may lead to a predisposition to immunologic diseases, yet none of the studies they cited pertain to pregnancy. However, one study was found in the literature that investigated the relation between vitamin D status during pregnancy and the subsequent development of immunologic diseases (1). This case-control study (85 cases and 1071 controls) from Norway found that cod liver oil taken during pregnancy was associated with reduced risk of type 1 diabetes in the offspring. These investigators, however, did not find an association between type 1 diabetes among the offspring and the prenatal use of multivitamin supplements, which typically contain significant amounts of vitamin D. This association, therefore, is not likely to be a direct result of maternal vitamin D status, but could be due to additional nutritive factors in cod liver oil or other confounding factors. Although the studies discussed by Reichrath and Querings are intriguing, it would be premature to state that there is evidence that vitamin D deficiency during pregnancy may be a predisposing factor for the fetus to the development of immunologic diseases.

The author had no conflicts of interest to report.

Bonny Specker
Dear Sir:

I would like to comment on the article “Iron in ferritin or in salts (ferrous sulfate) is equally bioavailable in nonanemic women,” by Davila-Hicks et al (1). The conclusion indicated in the title is based on measurements of iron absorption from horse spleen ferritin that was radioiodlated in vitro and appears to contrast with the results of others whose studies using ferritin radiolabeled in vivo were not cited (2–4). For example, Skikne et al (4) also found that iron from ferritin radiolabeled in vitro was absorbed similarly to iron from ferrous sulfate. However, the same group further reported that radiiodiron incorporated into bovine spleen ferritin in vivo was significantly less absorbed than was iron from ferrous sulfate: 3.2% compared with 8.2% from a 3-mg dose with food, 3.8% compared with 24.1% from a 3-mg dose without food, 0.6% compared with 2.6% from a 50-mg dose with food, and 0.7% compared with 7.9% from a 50-mg dose without food (4). Those who have studied ferritin radiolabeled in vivo have concluded that ferritin iron is poorly absorbed and that it is not part of the nonheme pool of dietary iron that is readily exchangeable in and is similarly absorbed from the intestinal lumen (2–4). For instance, in vivo–labeled ferritin 59Fe was only 36% as well absorbed as was 59Fe from intrinsically labeled soybeans consumed in the same meal (2). It is possible that a lower absorption of ferritin iron may explain the slightly greater (10%) absorption of nonheme iron from extrinsically than from intrinsically labeled foods (5), which suggests that the ferritin iron content of food is only a minor portion of total food iron. It is worth noting that the ferritin iron content of foods has not been widely determined because of the lack of species-specific antibodies as well as the insolubility and possible time-dependent molecular changes that may make ferritin iron less exchangeable (6).

Each labeling method has potential problems. On the one hand, the in vivo labeling of animal ferritin has in some (2, 4), but not in all (3), reports involved procedures to limit the radiolabel incorporation into blood by reducing erythrocyte synthesis or increasing erythrocyte breakdown, and it is not known whether these techniques alter ferritin isomerization. It is clear that the in vivo procedure does not uniformly label all of the iron in ferritin, but this would not necessarily explain the reduced iron bioavailability because the portion that is unlabeled may be less, not more, exchangeable or absorbable. On the other hand, in vitro labeling results in higher bioavailability regardless of whether the ferritin has first been depleted of iron (1) or not (4), and in vitro iron exchange can induce ferritin degradation through Fenton chemistry (6). Skikne et al (4) observed a minor small molecular peak in the Sepharose 6B elution pattern of in vitro, but not in vivo, labeled ferritin, that they proposed to be denatured ferritin. Those investigators (4) determined that in vitro procedures labeled a full range of isoforms but that isotope incorporation into the more acidic forms was slightly higher (4). It is unlikely that horse spleen ferritin labeled with extra phosphorus in vitro (1) is comparable with plant ferritin. Using Mössbauer spectroscopy, Ambié et al (7) found that the form of ferric iron, representing ≈95% of the iron in soybeans, was clearly distinguishable from, but more similar to, horse spleen ferritin than to ferric phytate. Although physicochemical methods detected only minor alterations in ferritin labeled in vitro (1, 4), the human absorption results provide a distinguishing bioassay for ferritin labeled in vitro compared with in vivo.

Davila-Hicks et al (1) proposed that a high absorption of iron from the Tokyo soybean cultivar is partially explained by a high ferritin content of this cultivar, in addition to the low iron status of the subjects (8). After logarithmic transformations of both variables, absorption of iron is inversely related to body iron stores, varying 10–15-fold between subjects (see Figure 1 of reference 9). This relation alone is sufficient to account for the differences in iron absorption from soybeans cited by Davila-Hicks et al (1): 26% in women with borderline iron deficiency (assuming 80% red blood cell incorporation of absorbed isotope) (8), 20% in women with iron deficiency (assuming 100% red cell incorporation; the absorption calculation is increased to 25% if red blood cell incorporation is assumed to be 80%) (10), and 2.8% in iron-replete men (11). Lacking a direct comparison of cultivars with the same subjects, the similar results obtained by Murry-Kolb et al (8) and Sayers et al (10) do not support the hypothesis that the iron from the high-ferritin Tokyo soybean cultivar was more bioavailable than was that from commonly used soybean cultivars.

Davila-Hicks et al (1) concluded that iron from ferritin or ferrous sulfate follow different metabolic pathways after absorption. This was based on similarities in iron absorption when measured by whole-body scintillation counting (22% and 22% from ferritin and ferrous sulfate, respectively; see Table 1) but differences in absorption when measured from erythrocyte iron incorporation (27% and 48%). The greater retention of isotope in the erythrocytes than in the whole body suggests methodologic difficulties. The specific method and assumptions used were not delineated. With the use of commonly used methods (see citations in reference 9) and an assumption of 80% incorporation of the absorbed isotope into blood, we repeatedly obtained similar absorption results between the 2 methods, including results with added ferrous sulfate (9). For instance, nonheme-iron absorption from a hamburger meal supplemented with 20 mg Fe as ferrous sulfate was 8.4% (geometric mean ± 1 SE: 6.8, 10.3) by whole-body counting (see data in Figure 1 of reference 9) and 8.5% (6.7, 10.7) by the erythrocyte incorporation method, and the assumption of 80% incorporation of the absorbed isotope into blood was confirmed (9). Note that the blood incorporation data in reference 9 was incorrectly labeled as the incorporation of the ingested rather than of the absorbed isotope dose; an erratum was submitted. Davila-Hicks et al (1) appear to have expressed the blood incorporation as a percentage of the ingested dose; expressed as such, their values seem excessive, but when expressed as the percentage absorbed seem insufficient.

With the use of data from individual subjects (n = 23), the blood incorporation method was highly correlated with the whole-body counting method (R2 = 0.98) (9). These data do not confirm the finding that iron from ferrous sulfate is more extensively incorporated into blood than is apparent from whole-body counting measurements.

In conclusion, research on iron bioavailability from ferritin labeled in vitro must be interpreted with caution. The evidence does not support the conclusion that iron absorbed from ferrous sulfate...
The discussion of the causes of the apparent inconsistencies in the results of using isotopically labeled ferritin in iron-absorption experiments appears in our first article on iron absorption from ferritin (5) and again in a recent review (6). Briefly, when labeled iron atoms are added to the unlabeled, solid mineral in ferritin, equilibration is very slow (days to months). In the 1997 study annotated in Hunt’s letter (7), the label was added to ferritin (bovine), which had endogenous iron both in vitro and in vivo. In addition, in that study the estimate of the iron:protein ratio in the isolated ferritin was very high, based on the data in the article, which suggested the presence of denatured ferritin protein/hemosiderin to which some labeled iron added in vitro may have been adsorbed, giving rise to the inappropriate high absorption observed (7). None of these possibilities would have been detected in the protein analyses described in the article by Hunt, because the effect is on the location of the labeled iron in the iron mineral.

The assertion in Hunt’s letter about the plant ferritin mineral in reconstituted horse spleen ferritin is incorrect. We previously compared the mineral in pea ferritin with that in horse spleen ferritin reconstituted to have a plant ferritin mineral composition and found, using EXAFS analysis, that the high phosphate mineral content in horse spleen ferritin was similar to that in natural pea ferritin (8) and distinct from that in animal ferritins; the work was annotated in our recent paper. For Hunt’s assertion to be correct, the nonconserved structural features would have had to dominate the many conserved structural features of ferritin protein in iron absorption. In our recent article, we were merely exploring whether the structure of the plant ferritin mineral influenced iron absorption in humans. Studies currently in progress will explore the influence, if any, of the plant ferritin protein on iron absorption. Hunt misunderstood our statement about soybean cultivars, which simply indicated that soybeans with more ferritin would likely have a greater proportion of the bean iron in ferritin. Under the hydroponic, nonnodulating conditions used in the study described (9), the percentage of bean iron in ferritin was lower than that in field-grown beans. All soybean cultivars have a large fraction of the iron in ferritin when grown under field conditions.

In contrast with the statement in Hunt’s letter, we did not “conclude” that iron from ferritin or ferrous sulfate follow different metabolic pathways after absorption, we merely suggested this as a possible explanation for the differences observed. Further molecular studies at a cellular level are needed to verify this, but our own preliminary data from Caco-2 cells (presented at Experimental Biology 2004) support such a scenario. We did not intend to state that the retention in erythrocytes was greater than that in the whole body. We used the conventional approach for estimating whole-body retention from red blood cell (RBC) incorporation, ie, assuming 80% incorporation and calculating whole-body retention from blood volume estimated from body weight. This was stated in the text as the well-known “RBC incorporation method for estimating iron absorption” rather than in explicated detail in the Methods, which, unfortunately, may have caused confusion. We obtained a higher absorption using this method than when using whole-body counting. In Hunt’s previous work (10), a lower value was obtained with the RBC method (calculated from Figure 1 in reference 10) than with whole-body counting. These differences between the 2 methods for determining iron absorption may be related to differences in the geometry of the counter when determining radioactivity from a point source (blood) rather than from a whole body. However, in our study, the same method was used in the 2 groups of subjects. We observed a difference for ferritin iron and ferrous sulfate, which suggested a difference in metabolic handling of the 2 types of iron. Further studies are needed to explore this in more detail.

To summarize, we showed an efficient absorption (20–25%) of ferritin iron in several studies using different analytic methods to
REFERENCES


Erratum


On page 1014, Table 3, the mean daily intakes of olive oil by men and women were incorrect, although there was no error in the corresponding SDs. The correct mean (±SD) intakes (in g) were 53.6 ± 24.0 for men and 46.4 ± 21.8 for women. A programming error was responsible for this overestimation.

The partial regression coefficients of systolic and diastolic blood pressure on SD increments in olive oil intake and the corresponding 95% CIs and P values (reported in Tables 4 and 5 and throughout the text) were unaffected by this error.
Books Received


Preface

Suboptimal intake of micronutrients is a significant public health issue for women of all ages, and has particular ramifications in women of childbearing years. Women’s micronutrient status affects not only their own health, but also the health of the next generation. Low intakes affect a wide range of health issues, including pregnancy outcomes, learning disabilities, bone development, immune function, and even neurological function in the elderly. A symposium, Women and Micronutrients: Addressing the Gap Throughout the Lifecycle, was convened by the Institute of Human Nutrition of Columbia University on June 6, 2004. The proceedings are summarized in this supplement.

The goals of the Women and Micronutrients symposium that led to this supplement were to provide awareness on the adverse impacts of the current gaps in micronutrient consumption by women of all ages, and to translate basic science findings related to micronutrient status in women into strategies for improved women’s health. The discussants provided a comprehensive overview of how poor micronutrient status translates to poor outcome at every stage of the life cycle, and reviewed and made recommendations in the following areas:

- A lifecycle micronutrient perspective for women’s health
- Dietary Reference Intakes: development and uses for assessment of micronutrient status of women: a global perspective
- The process of developing DRIs
- Multiple micronutrients in pregnancy and lactation
- The impact of folate status on pregnancy outcome and chronic disease in women
- Iron status during pregnancy: setting the stage for mother and infant
- Menopause, micronutrients, and hormone therapy
- The impact micronutrients have on osteoporosis
- Micronutrient requirements in older women
- Micronutrient requirements of physically active women and what we can learn from iron

It is widely acknowledged that highly nutrient dense food is a preferred vehicle for repletion of a micronutrient deficiency. However, it is also clear that there are substantial and often insurmountable problems with the logistics of providing such food nationally and worldwide. Additionally, the challenges regarding acceptance/compliance and access to such diets are also considerable. Until the logistical challenges of distribution, acceptance, and access are met, micronutrient supplementation should be considered as a viable means of filling the micronutrient gap in women throughout the life cycle.

Barbara A Underwood  
Richard J Deckelbaum  
Sharon R Akabas
A life cycle micronutrient perspective for women’s health

Kellee A Bartley, Barbara A Underwood, and Richard J Deckelbaum

ABSTRACT
Micronutrients not only benefit women’s health during childbearing years and during pregnancy and lactation, but they also have substantial impact on women’s health during adolescence and the aging years. Thus, for women, diet quality is important for health today and in the future. Realizing that there are many ways to improve the quality of a diet and to obtain adequate amounts of vitamins and minerals from foods, food-based approaches are still not attaining adequate intakes in most women, both in the United States and worldwide. Efforts are needed to improve diet quality, focusing on the diet as a whole, and not on single vitamins or minerals. However, consideration must be given to fortified foods and/or supplements to insure micronutrient adequacy.

KEY WORDS Nutrition, micronutrients, women, life cycle, n-3 fatty acids, folic acid, obesity, pregnancy, iodine, iron, prevention, food-based approach, fortification

NUTRITION THROUGH THE LIFE CYCLE

Worldwide, micronutrient status of women is inadequate for several micronutrients. Women’s micronutrient status affects not only their own health, but also those of the next generation, as depicted in Figure 1 (1). Micronutrients benefit women’s health during childbearing years, pregnancy and lactation, and they also have substantial impact on women’s health during adolescence and the aging years. The rationale for micronutrient adequacy in the individual woman has been well defined for many micronutrients such as iron, calcium, iodine, folate, and vitamins A and D. The timing of when adequate intake is critical is often different for a particular micronutrient. Adequate folate status, for example, will reduce the risk of congenital defects only pre-conception, and this is also generally true for iron in reducing the level of pregnancy anemia. In contrast, correcting iodine inadequacy during pregnancy has a marked impact on preventing the devastating effects of subclinical iodine deficiency on infant and childhood cognitive development (2), whereas too little vitamin A during pregnancy can impair maternal immunological protection as well as impair development of the infant’s immune system. During pregnancy and lactation, micronutrient adequacy for certain micronutrients such as iron can have substantial influence on pregnancy outcome, such as pregnancy mortality. Micronutrient adequacy will have impact on the ability of the mother to provide optimal feeding and lactation after delivery. An example of this includes prenatal n-3 fatty acid status, which affects the quality of human milk in terms of levels of these fatty acids after delivery (3-5). These long-chain n-3 fatty acids influence pregnancy and infant outcomes in terms of potential for learning and resistance to infections.

For the infant itself, it is increasingly realized that micronutrient inadequacies can be one of the contributors to low birth weight (6, 7). Low birth weight in the infant is not only a major cause of stunting in childhood and adulthood, but will also lead to increased risks for adult chronic diseases (8). Some of these risks can either be enhanced or diminished in low birth weight infants depending on feeding patterns during the first year.

At the other end of the life cycle, relatively little is known about the nutritional requirements of older women, especially those living well beyond their 80’s. Many of the normal processes of aging including acute and chronic illness, decline in organ function, and medications, all of which affect nutritional requirements. Multiple micronutrient deficiencies in elderly women are common (9-11). The limited information available on micronutrient requirements for elderly women is in large part due to the difficulty in conducting reliable and valid studies in this heterogeneous population that has various medication needs, different socioeconomic status, and different rates of aging and functional decline. Much benefit would be gained from additional research on the impact of better micronutrient status in young and middle age women on their health at older ages.

In macronutrient status, an increasing body of evidence indicates that maternal overweight and obesity before conception correlates to increasing risk of maternal complications during pregnancy such as gestational hypertension, gestational diabetes, need for delivery via Cesarean delivery, and need for hospitalization before delivery (Figure 2) (12). Very under-appreciated by many health professionals is the increasing body of evidence linking prepregnancy maternal overweight and obesity to increasing risk for birth defects. A number of publications now show close associations with maternal overweight with increasing risk of neural tube defects, as well as other neurological abnormalities, congenital heart disease, intestinal malformations, and multiple congenital anomalies (Figure 3) (13-16). Interestingly, for some of these defects, increased risk is already
maximal when the BMI is in the overweight range (25–25.9) with no further increase crossing over into the obesity range (BMI > 30) (16).

The importance of female nutrition precedes and extends beyond the reproductive years to optimize completion of adolescent growth and establish body nutrient reserves before pregnancy and to maintain adequate nutritional status, particularly skeletal health, through the postmenopausal years. Hence, the need for a constant, balanced, and adequate supply of all essential nutrients throughout a woman’s lifetime to optimize both her and her offspring’s health. This is best achieved safely through a consistent balanced intake of nutrient-rich food, including fortified foods, and nutrient supplements when necessary, and regular exercise.

Effects of micronutrient deficiencies

Though prevalence rates of micronutrient inadequacy vary considerably between developing and industrialized nations, the problem of micronutrient deficiencies is highly prevalent worldwide. Low dietary intake is the primary cause of micronutrient deficiencies at a public health level, but genetic factors, nutrient interactions, poor absorption, drugs, and diseases such as diabetes and hypertension may contribute, especially at an individual level. Among women of childbearing age, inadequate intakes of calcium, iron, folate, zinc, and vitamins A and D remain too common (17, 18). Among these, iron, folate, vitamin D, and zinc are of greatest concern due to high losses and requirements; these can be linked to an inadequate consumption of fruits, vegetables, meat, and animal sources of food. Even in Europe as a whole, we can still find prevalences of iron deficiency anemia in almost 22% of children up to 4 y of age, and 24% prevalence of iron deficiency in pregnant women (19). Prenatal and early pregnancy supplementation is necessary to minimize the risk of adverse pregnancy outcomes because replenishment of stores is difficult to achieve during pregnancy. Thus, iron inadequacy is still a major contributor to suboptimal outcomes for women throughout the life cycle and particularly for children, in terms of cognitive development, worldwide.

B-vitamin deficiencies are also of concern in the United States and abroad. Recent years have drawn attention to deficient folate intakes because of the relation to neural tube and other birth defects that can occur early in pregnancy. Adverse risks can be controlled at intakes of 400 μg daily, achievable through food, natural and folic acid fortified, and/or supplements. Vitamin B-12 deficiency is a problem now documented in several countries, particularly among those who do not eat meat and other...
animal products, such as lacto-ovo vegetarians and those on restricted meat intakes for access, economic, and/or cultural reasons (20).

Goiter, hypothyroidism, and cretinism are well-recognized consequences of severe iodine inadequacy; however, less recognized is the effect of milder levels of iodine inadequacy on poor reproductive outcomes such as stillbirths and birth defects (21). Outside of the nutrition community, there is also an underappreciation of the effects of “subclinical iodine deficiency” and decreasing cognitive achievement in children who will develop into adults with lower levels of intelligence than if they had had adequate iodine supply in utero (22). Similarly, mild levels of vitamin A inadequacy, insufficient to lead to night blindness or complete blindness, result in higher susceptibility to morbidity and mortality from infectious diseases as well as to adverse reproductive outcomes (23). In addition, restored vitamin A status improved hemoglobin levels in deficient populations, including pregnant women.

Thus, there are overlapping reproductive and development consequences of micronutrient inadequacies, including impaired cognitive development in learning ability, impaired immunity, adverse reproductive outcomes and maternal health problems, and finally, among infants and children, an abnormal bone matrix and distorted skeletal formations.

**Correction–prevention of micronutrient inadequacies**

Optimally, adequate intake of micronutrients should be achieved by food-based approaches. If successfully implemented, food-based approaches offer the following advantages:

- Focus on food groups, not single nutrients
- Focus on amounts, knowledge, and behavior
- Focus goes beyond foods (e.g., healthy weight)
- Varies portion size, not dose
- Reduces risks of high dose or the adverse interactions among nutrients
- Sustainability

However, dependence on food alone can have the following disadvantages:

- Availability, access
- Cost
- Interference by sociocultural factors
- Specific micronutrient adequacies may be difficult to achieve for certain nutrients (e.g., iron, folate)
- Decreased bioavailability with certain nutrients
- Inadequate compliance to dietary recommendations

Examining the micronutrient quality of the major staples of diets in a number of regions such as rice, wheat, or maize/beans, it is clear that the major staples themselves are by and large poor sources of many important micronutrients (Figure 4A) (24). But, if successive other foods are added, which increases diversity of the diet, adequacy of most micronutrients can easily be achieved as demonstrated in (Figure 4B) (24) for rice. In this example, the addition of one serving of carrots a day will provide recommended levels of vitamin A, and vitamin C adequacy will be met by a serving of oranges. If sufficient diversity can be made available and affordable, adequacy of many micronutrients can be achieved through food. However, in most populations, this is not the usual pattern because of individual or family choices, or lack of accessibility. Therefore, fortification with micronutrients is emerging as a powerful tool in preventing or correcting inadequacies for a number of micronutrients. Classical examples include fortification of salt with iodine, milk with vitamins A and D, and especially for children, infant formula and cereals fortified with iron and/or other micronutrients. In the United States,
Fortification of flour with folic acid is showing remarkable success in decreasing neural tube defects even though the level of fortification in the United States provides only about half of the recommended requirements for folic acid daily (25–27). In other countries such as Chile, fortification is at double the level of the United States and the results in terms of prevention of neural defects seem promising (28). The benefits of folic acid fortification/supplementation are not only applicable to women throughout the life cycle but also to all sectors of populations because of its ability to lower homocysteine levels and associated morbidity and mortality from cardiovascular disease (29, 30). High homocysteine levels are a risk factor not only for cardiovascular disease but are also related to adverse effects during pregnancy (31, 32), and perhaps even in neurological deterioration during aging (33). Folic acid is also emerging as important in lowering the risk of certain types of cancers (34) and perhaps independently neuropsychiatric disorders (35).

**Recommendations**

Realizing that there are many ways to improve the quality of a diet and to obtain adequate amounts of vitamins and minerals from foods, food-based approaches are still not attaining adequate intakes in most women, both in the United States and worldwide. Efforts are needed to improve diet quality, focusing on the diet as a whole, and not on single vitamins or minerals. However, at the same time, consideration must be given to fortified foods and/or supplements to insure micronutrient adequacy. To achieve these goals, the following are summary recommendations for women throughout the life cycle:

![Figure 4](image-url)

**Figure 4.** Nutrient density of common staple food (A): effects of sequentially adding small amounts (40-60g) of nutrient-dense foods to one example, rice (B). Data adapted from reference 24.
1. Provide guidance for selecting, combining, preparing and serving micronutrient-rich foods to improve diet quality using available information.

2. Improve access to and use of foods that are rich in vitamins and minerals, using methods that are compatible with local dietary patterns and cultural values.

3. Where it is not possible to meet all needs through available food sources including fortified foods, insure the availability of appropriate vitamin and mineral supplements for women.

Overall, a life cycle and holistic perspective is essential in the design of effective intervention approaches to prevent micronutrient undernutrition in women and their children.

CONCLUSION

A decade ago, the World Bank summarized that vitamin/mineral deficiencies deprive one billion people worldwide of their “intelect, strength, and vitality.” They estimated that for <0.3% of their GDP, micronutrient-deficient countries could rid themselves of this entirely preventable problem, which now is costing >5% of their GDP in lost lives, disability, and productivity (36). Thus, as we move into the 21st century, we can aim for new paradigms for micronutrient interventions, which would result in significant health improvement, better pregnancy and infant outcomes, and economic benefits worldwide. These paradigms include emphasizing:

- Prevention more than cure
- Promotion of healthy lifestyles
- Insuring micronutrient adequacy to periconceptional women and infants within a life cycle mode

Because adequacy of micronutrient intakes is often not achieved through food-based approaches and because initiation of fortification programs often takes years of planning and integration between private and public sectors, supplementation is often necessary in vulnerable groups to achieve micronutrient adequacy. There are multiple reasons for assuring adequate micronutrient status peripregnancy and throughout lactation to achieve optimal infant and young child outcomes and maternal health. Multiple micronutrient containing supplements are widely available and after initial survey for adequacy, women should be encouraged to take supplements when their needs are not met by food-based or fortification approaches.

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Dietary Reference Intakes: development and uses for assessment of micronutrient status of women—a global perspective

Eileen Kennedy and Linda Meyers

ABSTRACT
This paper reviews the process of developing the Dietary Reference Intakes (DRIs) and provides a synopsis of the micronutrient status of women worldwide. At a 1993 symposium organized by the Food and Nutrition Board (FNB) of the Institute of Medicine (IOM), it was decided that the Recommended Dietary Allowances (RDAs) would be replaced by the DRIs, which would address several issues that the RDAs did not, including chronic disease risk reduction, upper levels for nutrients where toxicity data existed, and the possible health benefits of some food components that did not meet the traditional definition of a nutrient. Another important distinction is that because the DRIs are comprised of 4 reference values—the Estimated Average Requirement (EAR), RDA, Adequate Intake (AI), and a tolerable Upper Level (UL)—and not a single reference value like the previous RDAs, they could be used to differentiate planning from diagnosis or assessment.

The latest DRIs and nutrient intakes are shown for iron, zinc, calcium, Vitamin A and folate status in women in the United States and Canada. Data on the micronutrient status of women globally are much more limited. Summary statistics on iron deficiency anemia, night blindness, and risk of zinc deficiency are summarized.

KEY WORDS DRIs, RDAs, EAR, UL, iron, zinc, vitamin A

INTRODUCTION
The RDAs were first published in 1941 and provided the scientific basis of nutrient recommendations for the US population. From 1941 to 1989, the RDAs were updated periodically based on newer scientific information on individual nutrient requirements. The impact of the RDAs were wide-ranging; the RDAs provided the nutritional standard for the US nutrition programs (1) and served as the basis for daily values (DVs) used in nutrition labeling (2). In 1993 a major revision of the RDAs was undertaken by the Institute of Medicine. The RDAs have been replaced by the DRIs, a set of nutrient-based reference values. The DRIs are now used in place of the RDAs in the United States as well as replacing the recommended nutrient intakes (RNIs) for Canada. This paper reviews the process of developing the DRIs, and implications for assessing the micro nutrient status of women worldwide for a range of nutrients.

DIETARY REFERENCE INTAKES
The process of developing the DRIs began in June 1993 with a symposium organized by the FNB of the IOM. The DRI initiative emerged from this meeting. The FNB concluded that there was sufficient new information to support a major reassessment of the RDAs (3). The recommendations emphasized that this reassessment should also address the concepts of diet and the relation to risk reduction for chronic diseases. The DRI initiative, for the first time, would consider the issue of upper levels of intake for some nutrients. In addition, food components that did not meet the criteria of traditional nutrients would be considered in the DRI initiative. The final conclusion from the 1993 symposium was that the FNB needed to devote serious attention to a new format for presenting the DRIs.

The DRI initiative evolved over a 10-year period. The process that followed the 1993 symposium provided a comprehensive, coordinated approach for developing and communicating the DRIs. The framework that emerged for guiding the DRI initiative was posited on 4 basic assumptions (4). First, there was general agreement that functional endpoints had been identified for a number of nutrients and that these endpoints were relevant for establishing nutrient requirements. Second, the advances in defining nutrients requirements provided a clearer delineation of the uses of the DRIs; the application of the DRIs would be able to differentiate planning from diagnosis or assessment. One of the strongest arguments for the DRI initiative was the concern regarding the inappropriate use of the RDAs and RNIs. Unambiguously the FNB noted, “The availability of only a single type of reference value in face of various needs has led to inappropriate applications” (3). Finally, given the global nature of agriculture, food and nutrition issues, harmonization of the nutrient recommendations from the United States and Canada seemed warranted. Thus, it was agreed that the DRIs would be applicable in the US and Canada.

Four reference values now make up the DRIs. The EAR is the daily value that meets the average requirement for healthy individuals in which a functional/clinical indicator of adequacy has been established (4). The EAR is the median of distribution for population requirements; therefore, half the population will fall below and half above this value. The EAR is the basis of deriving the RDA, and is used as the primary reference for assessing the adequacy of groups.

1 From the Friedman School of Nutrition Science and Policy, Tufts University, Boston (EK) and the Food and Nutrition Board, Institute of Medicine, Washington DC (LM).
3 Address reprint requests and correspondence to ET Kennedy, Friedman School of Nutrition, Tufts University, 150 Harrison Avenue, Room 135, Boston, MA 02111. E-mail: Eileen.kennedy@tufts.edu.
The DRIs is the estimate of the nutrient requirement that meets the needs of 97 to 98% of the healthy individuals. The DRAs are intended to be a goal for the daily intake of an individual. The DRAs is not an appropriate value for assessing nutrient adequacy of groups because it covers the vast majority of the nutrient requirements of the population. When the requirement for a nutrient is normally distributed, the RDA is 2 standard deviations above the EAR. However, for nutrients that have a non-normal distribution other approaches are used. For example, to set the RDA for iron, a factorial modeling approach is used. This method uses the distribution of the individual requirements for a particular nutrient for age/sex groups. For example, iron requirements for infant, children, and pregnant women include requirements for basal losses as well as the amount needed for maintenance of growth and fetal development.

When insufficient data are available to calculate an EAR, a reference value called the AI is provided instead of the RDA. AIs are based on observed or experimentally determined approximations of nutrient intake by a defined population or subgroup that appear to sustain a defined nutritional status. More judgment is used in the calculation of an AI. In addition, the AI may be based on a single study. Similar to the RDA, the AI can be used as a guide to nutrient intake for an individual. In general, AIs are estimated to be above the RDA for a specific nutrient, although there may be some nutrients where this is not the case.

For the first time the DRI process involves the calculation of a tolerable UL for some nutrients. The UL is the highest level of daily nutrient intake that is likely to pose no risks of adverse health effect to almost all individuals in the general population. The DRIs are clear that the UL is not a recommended level of intake, nor is it a level that is desirable to attain. ULs have been developed through a risk assessment method.

The 4 DRIs, were established for 22 distinct life stages and sex groups. The life stages groups include: infants birth to 6 mo; infants 7 to 12 mo; toddlers, 1 to 3 y of age; early childhood, 4 to 8 y of age; puberty, 9 to 13 y of age; adolescence, 14 to 18 y; young adulthood, 19 to 30 y; middle ages, 31 to 50 y; adulthood, 51 to 70 y; older adults, age 70 and over; and pregnancy and lactation.

In addition to considering life stages and sex groups, the DRIs specified a reference body size to use when more specificity is needed regarding body size and nutrient requirements. For a male 19 to 30 y, a BMI of 22.4 is the reference. For women, in the same age category, a BMI of 22.8 is used. Clearly in the United States, the majority of adults—both men and women—have an average BMI well above the established reference body size.

The new DRIs are more complex than the RDAs and RNIs. In place of a single nutrient value, there are now 4 reference values. The DRIs employ a much broader conceptual approach. Indeed, the UL as a concept of risk had not been used in the RDA process.

The DRIs serve a variety of purposes. One function of the DRIs is to determine adequacy. Each EAR and AI in the DRIs is described in terms of a selected criterion or indicator of adequacy. For example, the AI for calcium is based on balance studies and calcium deposition in the bone. The determination of the EAR or AI for a particular nutrient is sensitive to the indicator chosen.

The DRIs have many uses. The DRIs are the nutritional basis for the Federal nutrition programs. A committee of the FNB recently reviewed the use of the DRIs for nutrition labeling (5).

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using the RDA for group estimates of nutrient adequacy. The RDA overestimates the level of deficiency for population comparisons.

Zinc is a nutrient that has garnered increased attention in the past decade. Similar to iron, the EAR for zinc is calculated using the factorial method. The RDA for women for zinc is 8 mg/d. The UL for zinc is 40 mg/d and is based on a reduction in erythrocyte enzyme activity. The EAR for zinc for women 19 to 70 y of age is 6.8 mg/d. Zinc data from the National Health and Nutrition Examination Survey (NHANES) for women 19 to 50 y of age is presented in Table 3. Here again, similar to iron intake data, zinc consumption either from food alone or food and supplements for each age group is above the EAR.

The EAR for vitamin A is based on the assurance of adequate stores. The RDA for women is 700 μg of retinol activity equivalents per day (RAE/d). RAE conversion factors are 1:12 for β-carotene, 1:24 for alpha carotene and 1:24 for beta cryptoxanthin. These conversion factors are much higher than the 1:6 conversion rates that had historically been used. The new conversion factors mean that larger amounts of provitamin A carotenoids are needed to provide the EAR for vitamin A. The EAR for vitamin A is 500 μg RAE/d. The UL for vitamin A for 3000 μg/d preformed vitamin A; worth noting is the fact that many dietary supplements currently available have vitamin A levels at or above this amount (9). The EAR for women 18 and older is 500 μg RAE/d.

The mean intakes of vitamin A for women are shown in Table 4. Women from 19 to 50 y of age have usual vitamin A intakes provided from food that is above the EAR of 500 μg RAE/d. In addition, the vitamin A provided by supplements to women in the 50th percentile of intake is an additional 1422 μg RAE/d; this is almost 3 times the EAR.

Calcium is a nutrient that was included in the first edition of the RDAs and low intakes of calcium continue to be a problem for subsets of the US population, in particular, female adolescents (10). The trends in calcium intake for adult women, 20- to 70- y-old, between 1971/74 and 1999/2000 are shown in Table 5. Calcium intakes have increased since the late 1980s in adult women and appear to be due to a combination of factors. Nutrition promotion campaigns targeted to women emphasizing the calcium/osteoporosis prevention theme and the addition of calcium to nontraditional food sources such as orange juice appear to be having some influence on calcium consumption patterns in women (8).

Folate has been identified as a shortfall nutrient in US adult women. In 1998 the Food and Drug Administration (FDA) mandated that all grain products be enriched with folate. This resulted in white bread, for example, increasing its folate content from 34.0 μg/100 g to 95.0 μg/100 g (11). Data on the serum and red blood cell folate before and after the change in bread and flour folate levels are provided in Table 6. For women aged 15 to 44 y of age, there are increases in both serum and red blood cell folate levels (12). There have been major improvements in the micronutrient status of women post World War II in the United States. A variety of policies and programs have contributed to this improvement, including US fortification policy, which has had significant positive impacts on vitamin A, iron, and the B vitamins (13). The increasing incomes of the US population over the past 50 y combined with an agricultural policy that has resulted in lower food prices has narrowed the gap in dietary patterns between low income and other income groups (13). Federal programs such as Women, Infants, and Children (WIC) have also contributed to improved micronutrient status in women (14).

MICRONUTRIENT STATUS IN WOMEN WORLDWIDE

In the early 1990s the problem of “hidden hunger”—micronutrient deficiencies—was given worldwide attention from a series of high level conferences, including the 1992 International Conference on Nutrition (15). It became apparent that large parts of the developing world were plagued by micronutrient malnutrition that could not be seen but that had devastating health and nutritional consequences. The initial focus on hidden hunger emphasized 3 micronutrients—iron, iodine, and vitamin A. The number of individuals worldwide affected by hidden hunger is staggering. Globally, some 250 million children are at risk of vitamin A deficiency, 1.5 billion people live in areas where iodine deficiency disorders are a threat, and 2 billion people suffer from anemia or iron deficiency (16).

Unfortunately, statistics on the micronutrient status of women in developing countries is much less available. Data have concentrated on preschool-aged children and the population as a whole. Information on women is less available and incomplete. In a recent report

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<td>Age Group</td>
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<tr>
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<td>11%</td>
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<td>20–49 y</td>
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<td>1988–1994</td>
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<td>1988–1994</td>
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<td>Calcium (mg)</td>
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<td>1971–1974</td>
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<td>599</td>
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<tr>
<td>1988–1994</td>
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<td>1999–2000</td>
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1 Reference 8.
released by United Nations Children’s Fund (UNICEF) and the Micro Nutrient Initiative, there is a sobering wake up call.

“... In the several decades since measurement of iron deficiency began, very little headway has been made against this problem... For many decades this has been seen as a “women’s problem”... affecting up to three-quarters of pregnant women. Among policy makers, the unspoken view has seemed to be that women somehow cope and that iron deficiency is not a high enough priority to justify a major nutritional effort to reduce it.” (16).

Data from selected countries on the percent of women, 15- to 49-y-old who suffer from iron deficiency anemia (16) are provided in Table 7. In each of these countries significant factors causing the anemia are low iron intakes and poor bioavailability of dietary iron. Most countries do not have nationally representative surveys of consumption patterns or micronutrient status. A crude estimate is that 500 million women ages 15 to 49 worldwide or 40% of females in the developing world are anemic (17). > 1000 severely anemic young women die every week because they lack sufficient iron to cope with the stress of childbirth (16).

Limited data are available on night blindness in pregnant women; prevalence rates for night blindness range from a low of 3.8% in the United States to 4.4% in Africa and 10.9% in South and Southeast Asia. Here again, the rates of night blindness, particularly in Asia, suggest dietary patterns chronically low in vitamin A.

Zinc is a nutrient that has only recently received increased attention. An international task force on zinc recently concluded that iron deficiency and zinc deficiency often occur in tandem (18). This task force notes that iron and zinc have a similar affective patterns or micronutrient status. A crude estimate is that 500 million women ages 15 to 49 worldwide or 40% of females in the developing world are anemic (17). > 1000 severely anemic young women die every week because they lack sufficient iron to cope with the stress of childbirth (16).

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Some general conclusions can be drawn from limited data on the micronutrient status of women. Micronutrient status is inadequate worldwide, and not surprisingly, the problem being more severe in low-income women (17). The poor micronutrient status is part of the larger problem of poor nutritional status, in general, in women.

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Micronutrient deficiencies and gender: social and economic costs1–3

Ian Darnton-Hill, Patrick Webb, Philip WJ Harvey, Joseph M Hunt, Nita Dalviy, Mickey Chopra, Madeleine J Ball, Martin W Bloem, and Bruno de Benoist

ABSTRACT
Vitamin and mineral deficiencies adversely affect a third of the world’s people. Consequently, a series of global goals and a serious amount of donor and national resources have been directed at such micronutrient deficiencies. Drawing on the extensive experience of the authors in a variety of institutional settings, the article used a computer search of the published scientific literature of the topic, supplemented by reports and published and unpublished work from the various agencies. In examining the effect of sex on the economic and social costs of micronutrient deficiencies, the paper found that: (1) micronutrient deficiencies affect global health outcomes; (2) micronutrient deficiencies incur substantial economic costs; (3) health and nutrition outcomes are affected by sex; (4) micronutrient deficiencies are affected by sex, but this is often culturally specific; and finally, (5) the social and economic costs of micronutrient deficiencies, with particular reference to women and female adolescents and children, are likely to be considerable but are not well quantified. Given the potential impact on reducing infant and child mortality, reducing maternal mortality, and enhancing neuro-intellectual development and growth, the right of women and children to adequate food and nutrition should more explicitly reflect their special requirements in terms of micronutrients. The positive impact of alleviating micronutrient malnutrition on physical activity, education and productivity, and hence on national economies suggests that there is also an urgent need for increased effort to demonstrate the cost of these deficiencies, as well as the benefits of addressing them, especially compared with other health and nutrition interventions. Am J Clin Nutr 2005;81(suppl): 1198S–1205S.

KEY WORDS Micronutrients, micronutrient deficiencies, vitamins, minerals, vitamin and mineral deficiencies, cost-effectiveness, cost-benefits, gender, sex, women, children

INTRODUCTION
Micronutrient deficiencies are so important to public health outcomes, particularly in the developing world, that a series of global goals have been established, and significant amounts of donor and national funds have been directed at them. A recent report highlights the magnitude of the problem and attempts to demonstrate the economic and health costs of vitamin and mineral deficiencies through a series of country-specific reports (1). It also demonstrates the cost-effectiveness of known micronutrient interventions and the need for greater funding. To achieve the Millennium Development Goals (2), improving the status, health, and welfare of women will be critical (3). Women comprise the majority of the world’s poor (4). In poor households, women play a critical role in ameliorating the effects of poverty, especially for infants and young children. Clearly, the reduction of micronutrient deficiencies, given that they have an impact on infant and child mortality; maternal morbidity and mortality; and development, growth, and economic and social well-being, needs to be aggressively tackled, not least to reflect the legal human right of women and children to adequate nutrition, including micronutrients.

However, given the emphasis particularly by most donor countries on economic rationalization of the past recent decades, there has also been a consistent call to demonstrate the cost benefit of programs addressing micronutrients, especially compared with other health and nutrition programs. The assumption that such interventions are cost-effective has heavily relied on statements from the World Bank which suggested that the cost of micronutrient deficiencies might be up to 5% gross national product (GNP) whereas interventions might only cost 0.3% of the GNP (5). A recent report, the “Copenhagen Consensus” resulted from economists setting priorities among a series of proposals for confronting ten major global challenges, by prioritizing the use of a hypothetical $50 billion made available to governments in developing countries. Providing micronutrients through a combination of public health and private sector programs was ranked second, after control of human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) (6).

This article explores the overall social and economic impact of micronutrient deficiencies by identifying and systematically bringing together available information on: (1) micronutrient deficiencies and health outcomes; (2) micronutrient deficiencies

1 From the UNICEF Nutrition Section & Institute of Human Nutrition, Columbia. University, New York (IDH, ND); the World Food Program (PW), Rome, Italy; MOST, the USAID Micronutrient Program/Johns Hopkins Bloomberg School of Public Health (PWJH); the John Guggenheim Memorial Foundation (Nutrition and Economics) (JMH); the University of the Western Cape, South Africa (MC); Human Life Sciences, University of Tasmania, Launceston, Australia (MIB); Helen Keller International, Singapore (MWB). Micronutrients Unit, World Health Organization, Geneva, Switzerland (BdB).


3 Address reprint requests and correspondence to I Darnton-Hill, UNICEF Nutrition Section, 3 U.N. Plaza, Nutrition Section (7th floor), New York, New York 10017. E-mail: idarntonhill@unicef.org.
and economic costs; (3) health and nutrition outcomes and sex; (4) micronutrient deficiencies and sex; and finally, (5) the social and economic costs of micronutrient deficiencies, with particular reference to women and female adolescents and children. Conclusions are then proposed along with policy and programmatic implications. The background information was drawn from the experience and information available to the authors, various reports, especially from multilateral agencies and by literature reviews using the key phrases micronutrients, vitamins, sex, women, socioeconomic status and cost. Most of the information comes from lower income country data, with limited information from socially disadvantaged populations in more affluent countries.

MICRONUTRIENT DEFICIENCIES AND HEALTH OUTCOMES

The adverse effects of micronutrient deficiencies and excesses in children up to reproductive age and beyond are well known and well documented, although some questions inevitably remain. The adverse effects include both functional and health outcomes involving growth and development, mental and neuromotor performance, immunocompetence, physical working capacity, morbidity, mortality, and overall reproductive performance and risk of maternal death (7). Affecting the size of the health impact are nutrient-to-nutrient interactions of micronutrients, age, sex, and other host and environmental conditions such as pregnancy, genetics, overall nutrition, infections, and social conditions such as economic status. For the purposes of this article, it is only necessary to point to the extensive evidence base of established reviews. All the micronutrients of public health importance have also undergone re-positioning with regard to their public health impact over the last several decades. The Global Burden of Disease estimates showed that among the 26 major risk factors of the global burden of disease (8), iron deficiency ranks ninth overall, zinc deficiency is eleventh, and vitamin A deficiency, is thirteenth (Figure 1).

Iron deficiency remains a public health challenge despite its long-recognized negative impact on the health and productivity of women (and of adult men). Its role in impairing the cognitive development in infants and young children has provoked a renewed interest in treating and preventing iron deficiency, although questions of effective and safe delivery remain (9). Iron deficiency in the 6–24 mo age group is impairing the mental development of 40%–60% of the developing world’s children (1). Widespread iron deficiency negatively impacts on national productivity with losses of up to 2% of the gross domestic product (GDP) in worst affected countries (1). Iodine deficiency in pregnancy is causing as many as 20 million babies per year to be born mentally impaired. This has been estimated to lower the average IQ of those born in iodine-deficient areas by 10–15 IQ points, which then adversely affects school performance, decreases productivity, and results in an enormous economic burden to nations (1, 10). Vitamin A is recognized as a major factor in reducing excess mortality from infectious diseases in developing countries, while deficiency remains the commonest cause in some countries of preventable childhood blindness (11). Its importance in public health terms has become more apparent in terms of a likely role in women’s health (12) and its elimination is a major 2010 UN goal (13). Zinc has recently been established as both important for the treatment of diarrhea but likely to have a role, along with other micronutrients, in prevention of both diarrhea and respiratory diseases (14). Folate has long been known to be important in the etiology of neural tube defects and anemia, but the role of folic acid has now been expanded to the prevention of cardiovascular disease, and as an essential component of flour fortification in most countries with fortification (15).
Given that single micronutrient deficiencies rarely occur in isolation, the public health importance of other micronutrients such as vitamin B12, riboflavin, and the role of multi-micronutrient formulations are receiving increasing attention (16). Approximately 70% of US pregnant women take multi-micronutrients as recommended medical practice, and there seems no clear reason why women in low- and medium-income countries should be denied the presumed benefits of this, especially if the dosage is given at no more than one recommended daily allowance (RDA). Higher doses need further investigation, especially in high HIV-endemic areas (17, 18), but may well have a role in delaying progression of HIV infection to AIDS (17).

MICRONUTRIENT DEFICIENCIES AND ECONOMIC COSTS

Developing countries are emphasized in this article because vitamin and mineral deficiencies are both highly prevalent in developing countries and because such deficiencies have major negative biomedical outcomes. However, there is also evidence that vitamin and mineral deficiencies are still highly prevalent in developing nations (such as China, Indonesia and Vietnam) where the availability of staple foods (and thus energy deficiency) are no longer problems. Some evidence shows vitamin and mineral deficiencies continue to be prevalent among lower income population groups in developed countries, such as the United States (19) and Europe (20). Karp (19), points out that, of the over 13 million children in the United States whose families live below the poverty level, ≈10% have overt micronutrient malnutrition.

In developing countries, intakes of expensive animal-derived foods are often not accessible to the poor and this substantially reduces intake of vitamins and minerals (21), whereas in industrialized countries, the poor diets in lower socioeconomic groups affect micronutrient intake more through low intakes of fruits and vegetables (20). In the Philippines, Bouis (22) showed that vitamin A deficiency was associated more strongly with a lack of knowledge than with low income, in contrast to iron deficiency, which was mainly economically determined. In pregnant women in rural Tamil Nadu, women’s intakes leading to low micronutrient intakes were most affected by eating customs and socioeconomic status (23). Poor people are more likely than others to suffer from micronutrient malnutrition, but micronutrient intake does not necessarily improve in step with increasing income (5). However, with increasing improvement in the quality of the diet, micronutrient status will generally improve.

As part of the development of the “PROFILES” package, Ross and Horton (24) developed algorithms for estimating the economic costs of anemia due to cognitive delays in children, lower productivity among adults, and premature births. The analysis suggested that the median value of productivity losses due to iron deficiency was about $4 per capita or 0.9% of GDP (24). On a per capita basis, losses are greatest in rich countries, where wages are higher, even though iron deficiency is less widespread. Nevertheless, the estimated cost to South Asia, where the prevalence of anemia is highest, was estimated to be around $5 billion annually (Figure 2). The dominant effect for all countries is the loss associated with cognitive deficits in children (24). Horton, using an econometric model, estimates that just 3 types of malnutrition—protein-energy malnutrition, iron deficiency and iodine deficiency—are responsible for 3%–4% of GDP loss in Pakistan in any given year and 2%–3% of GDP loss in Vietnam. Maternal anemia is responsible for 20%–22% of maternal deaths due to complications of pregnancy and unsafe birthing situations (25). Productivity of adult anemic agricultural workers (or other heavy manual labor) is reduced by 1.5% for every 1% decrease in hemoglobin (Hb) concentration below the established threshold for safe health (26) (see Figure 2).

The World Bank summarized the benefits of micronutrients in terms of cost per life saved and productivity gained per program (Table 1). For saving lives at least cost, targeted supplementation to at-risk groups (pregnant mothers for iron, under-fives for vitamin A) is more cost-effective than fortification, although the latter is a more sustainable solution in the long run as incomes rise and households gain access to higher-quality primary health care.
TABLE 1
Costs and benefits of micronutrient interventions: returns on nutrition investments

<table>
<thead>
<tr>
<th>Deficiency/Remedy</th>
<th>Cost per life saved (US$)</th>
<th>Discounted Value of productivity gained per program (US$)</th>
<th>Cost per DALY gained (US$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron deficiency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supplementation</td>
<td>800</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>of pregnant women only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortification</td>
<td>2,000</td>
<td>84</td>
<td>4</td>
</tr>
<tr>
<td>Iodine deficiency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supplementation (repro-aged women only)</td>
<td>1,250</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>Supplementation (all people under 60)</td>
<td>4,650</td>
<td>6</td>
<td>37</td>
</tr>
<tr>
<td>Fortification</td>
<td>1,000</td>
<td>28</td>
<td>8</td>
</tr>
<tr>
<td>Vitamin A deficiency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supplementation (under 5 only)</td>
<td>325</td>
<td>22</td>
<td>9</td>
</tr>
<tr>
<td>Fortification</td>
<td>1,000</td>
<td>7</td>
<td>29</td>
</tr>
<tr>
<td>Nutrition Education</td>
<td>238</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nutrition education and maternal literacy</td>
<td>252</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 D Aly = disability-adjusted life year.

care. Nevertheless, properly targeted supplementation is justified while fortification programs are in the early stage and expanding coverage, as long as the targeting principles reflect risk assessment and are consistently applied. Food-based interventions have the potential to be the most sustainable interventions for micronutrient deficiencies, although they are unlikely to be sufficient in the short term in poverty and emergency settings. The multiple benefits of food-based approaches are clear but rarely factored in. They include increases in intake of many nutrients, improved food security, female empowerment, and increased cash incomes that are likely to be spent on children’s nutrition and girls’ education. An observational study in rural India found that the micronutrient-rich food consumption by pregnant women, specifically that of milk, green-leafy vegetables, and fruits, were independently associated with the size of the infant at birth (23).

In addressing the returns on investments to reduce micronutrient deficiencies, Hunt (27) has concluded the following for the 4 micronutrients of public health interest.

Iodine

By eliminating iodine deficiency in previously iodine-deficient areas, the average economic gain produced by the increase in cognitive development is similar to the average economic gain in preventing a low birth weight child, with a benefit-cost ratio, per deficient women, of greater than one (28). Studies from Germany, India, Latin America, and the United States have shown the benefits of different interventions to reduce iodine deficiency (10). In Ecuador, people with moderate deficiency were consistently paid less for agricultural work (Greene 1977 cited in 26). More recently, WHO looked at the cost-effectiveness and benefit-to-cost ratios of micronutrient interventions, especially fortification and salt iodization programs; both of which were identified as having very high benefit-to-cost ratios (29). Depending on the assumptions made, the benefit-to-cost ratio is from 40 to 400:1 (10). The mortality risk associated with iodine deficiency is the least well-known; limited results indicate a possible 8% benefit in child mortality reduction (30).

Vitamin A

Meta-analyses of field trials of mass vitamin A supplementation to children 6 to 59 mo of age have indicated an overall reduction in child mortality by 25%–35%, despite less consistency in the rates of reduction in morbidity, with greater reduction in severity than incidence of illness (31). Impacts on morbidity are also mediated by presence or absence of other deficiencies such as iron and zinc. In contrast to low birth weight, the benefits from the productivity gains over decades of work (from preventing blindness) are relatively small in comparison to benefits from reducing early mortality. A series of careful country studies on costs of vitamin A supplementation programs for under-5-year-old (Ghana, Guatemala, Nepal, Peru, and Philippines), revealed that the unit costs of the vitamin A supplement ($0.04 per child for 2 doses) represent 5% of the delivery costs when the full program is costed (32). Though additional therapeutic benefits have regularly been noted in clinical settings, e.g. the now standard practice of providing vitamin A to children suffering from measles and the use of vitamin A to treat clinical signs of xerophthalmia, the estimation concentrated on the benefits from the prophylactic provision of vitamin A. The worldwide average is now estimated to be $1.50 for 2 doses of vitamin A per child per year, highly cost-effective given the role of vitamin A deficiency as a primary risk factor in infant and child mortality. Eliminating vitamin A deficiency would save 16% of the global burden of disease in children (30).

Iron

The estimation of the benefits of reducing iron deficiencies must incorporate the fact that iron deficiency anemia can affect adult worker productivity directly, as well as through its impairment of child development. Behrman and Rozenweig (28) assumed a 5% across the board loss of labor productivity due to current anemia in addition to the gains through reducing low birth weight. However, increased benefits come at increased costs, because to obtain these additional ongoing productivity gains, there must be continued interventions over the work life in addition to the one-time intervention to reduce the number of low birth weight infants being born by antenatal supplementation to mothers. Eliminating severe anemia in pregnancy is estimated to potentially reduce the maternal disease burden by some 13%.

Zinc

Daily supplementation with zinc at home has been shown to reduce infant mortality by 70% and it is now recommended treatment for diarrhea, along with oral rehydration therapy (14, 33). There is little reported experience on delivery mechanisms suitable for large scale interventions (except, perhaps, with multiple fortification).

Noting that potential investments appear under-resourced, Behrman and Rozenweig have also noted the high rates of benefit-to-cost ratios and that the ‘gains appear to be particularly large for reducing micronutrient deficiencies in populations in
which prevalences are high” (28). The portion of the global burden of disease (mortality and morbidity, 1990 figures) in developing countries that would be removed by eliminating malnutrition is estimated by Mason, Musgrove, and Habicht as 32% (30). This includes the effects of malnutrition on the most vulnerable groups’ burden of mortality and morbidity from infectious diseases only. This is therefore a conservative figure, but nonetheless much higher than previous estimates, mainly due to now including micronutrient malnutrition (30). Seen in relation to the overall disease burden (all population groups, all causes, all developing countries), eliminating micronutrient malnutrition (in children plus anemia in reproductive age women) would save 18% of the global burden of disease, with eliminating child underweight an additional 15% (30).

HEALTH AND NUTRITION OUTCOMES AND SEX

Women and young girls are disadvantaged in health outcomes in the developing world whereas this may not be the case in the more industrialized world where women routinely outlive men. This does not preclude the possibility of social disadvantage, and an excess of some diseases such as depression (4). In most affluent countries, being a single mother is a strong risk factor for poverty or socioeconomic disadvantage. Globally, being a single mother or widow, and thus heading a female-headed household, almost invariably results in reduced income and increased likelihood of living in poverty (34). In their sample of South African female-headed households though, Lemke et al found that this did not necessarily mean they were less likely to have adequate food (34).

That many women are systematically discriminated against, or that a lower value is placed on women in many societies, is indisputable based on routine statistical indicators such as female-to-male life expectancy, and literacy (3) and with evidence, especially from South Asia, that they have less control over economic resources, than women in Norway or even Latin America (35). Regional differences in low birth weights may also reflect women’s status. This is important because low birth weight is the best single predictor of malnutrition (and likely some key limiting micronutrients) because it is associated with poor growth in infancy and throughout childhood (36), and increased subsequent mortality. Low birth weight may also lead to increased obesity and noncommunicable disease morbidity and mortality later in life (37). Maternal mortality ratio is a shocking 50 and 80 times higher in South Asia and sub-Saharan Africa than in the United States or Europe (38).

Sex disparities are reflected throughout the life course. While antenatal sex identification is increasingly performed, as in China and South Asia, there is a striking imbalance in male-to-female birth ratios (3, 39), presumed to be due to female feticide. The differences in ratios of girls to boys having primary education are well documented and vary strongly across countries. The adult literacy rate in South Asia for women as a percentage of those for men is 62%, compared with 72% in the Middle East and North Africa and in sub-Saharan Africa (13, 36). Across differences in wealth, not only is it harder for a girl living in impoverished circumstances to get primary education, but even if she does receive it, it is likely to be of shorter duration. She is more likely to be pulled out of school for family needs, and less likely, in most countries, to go onto higher education (3).

It has been pointed out—eg, by the UN Secretary General—that women are being particularly severely impacted by the HIV/AIDS epidemic as they are biologically, socially, and culturally more HIV-susceptible than men. HIV rates are 20% higher than men in sub-Saharan Africa, and much higher in younger age groups, with nearly 60% of those living with HIV/AIDS in sub-Saharan Africa being women (40). They are also less likely to avail themselves of health services for the treatment of opportunistic infections and more likely to forego food consumption in their household than men (41).

In settings that experience little nutrition improvement despite economic growth, social discrimination against women is common (42). In Pakistan, for example, widespread discrimination against girls and women is high and child malnutrition rates are among the highest in the world, as is the proportion of low birth weight infants, at 25%. Meanwhile in Thailand, where nutrition has improved remarkably in the last 2 decades, women have very high literacy, high participation in the labor force, and a strong place in social and household-level decision-making. Within India, women have similarly better relative status in Kerala compared with other states, and Kerala has better health, social and nutrition indicators, and not coincidently, the highest levels of female education (42).

MICRONUTRIENT DEFICIENCIES AND SEX

As previously noted, the evidence that infant, young child, adolescent, and adult females have significantly worse health and nutrition is strong, while this depends on the region concerned and social and status factors (35). There is surprisingly little information on micronutrient deficiencies and their relation to sex, although assumptions of sex discrimination are common. A recent review noted that while it is important to recognize that important differences do exist in prevalence rates for various micronutrient deficiencies by sex, physiology also plays a role in the expression of deficiencies, but such differences are not easily generalized (43). For example, 3 broadly accepted “facts” are often repeated in the micronutrient literature. The first is that boys are “at greater risk of xerophthalma (night blindness and Bitot’s spot) than are girls” (11, 44). The second is that women of reproductive age suffer a higher prevalence of iron deficiency than men do (45). The third is that “girls have a higher prevalence [of iodine deficiency] than boys,” especially from adolescence onward (46).

There certainly is evidence to support such claims. Vitamin A deficiency is commonly reported to be up to 10 times more common in males than females (44). Similarly, there is a well-documented higher risk of anemia in women of reproductive age due to menstruation and repeated pregnancies (45), with pregnant women at greater risk of being iron deficient when anemia is identified as a clinical manifestation (25). That said, few recent studies confirm empirically that the vitamin A status of boys is significantly lower than that of girls, but that seems to depend on the environmental, epidemiologic, and disease profiles of communities, as does iron deficiency anemia among men and children between 5 and 18 y of age (43). Indeed, most studies tend to assess micronutrient status of children without disaggregating by the sex of child, and focus on the status of mothers without considering the status of fathers or sons. The undifferentiated aggregation of people into broad categories of “children” or “women” can obscure wide variation in conditions as individuals...
Examples of micronutrients linked to sex disadvantage include the significantly higher risk of mortality among night-blind women compared with non-night-blind women even after the end of the pregnancy and the resolution of night blindness (38). Anemia affects 50%–70% of women during pregnancy and in severe forms will increase the risk of maternal mortality by up to 20% (1, 9). In a review on iron intake from India, it was reported that the intake of iron was <50% of the RDA for children 1–6 y old. For pregnant and lactating women, the intake was 37% and 49% of RDA respectively (47). In South Asia, and other areas where portions of the population are living in poverty, multiple micronutrient deficiencies coexist (16). It has been noted that even when females [in Asia] are apparently meeting energy and protein needs, they may still be at risk of micronutrient malnutrition due to lower intakes of more expensive animal foods, fruits, and treats of higher nutrient density (23). Nevertheless, the recent review by Webb et al (43) suggests that generalizations are not possible due to an enormous geographic and cultural variation, even for India.

In Bangladesh, this sex discrimination can actually have an unexpected impact in that while girls may receive a less favored diet, this might mean more dark green leafy vegetables so their levels of vitamin A are better than comparable boys and the levels of vitamin A deficiency less (43). Research from Mexico, however, showed no significant sex differences in dietary quality or quantity in infants and preschoolers even under conditions of economic and demographic stress (48). Nevertheless, school girls consumed significantly less energy per day than boys and less of all micronutrients examined, presumably because of lower total dietary intake. The authors concluded that the lower food intakes of girls did not appear to be due to purposeful diet discrimination, but rather to culturally patterned sex roles involving lower activity (48).

SOCIAL AND ECONOMIC COSTS OF MICRONUTRIENT DEFICIENCIES BY SEX

There is insufficient information on the costs of micronutrient deficiencies to people as individuals and communities. Using the PROFILES software package (Academy for Educational Development), estimates have been made for several countries. A recent example is Sierra Leone, where it was concluded that in the absence of adequate policy and program action to reduce anemia rates in women, the monetary value of agricultural productivity losses associated with anemia in the female labor force over the next 5 y would exceed $94.5 million; the present value of the future productivity losses associated with the intellectual impairment resulting from intrauterine iodine deficiency exceeds $42.5 million; and over 38 000 deaths of Sierra Leonean children under 5 y of age will be attributable to vitamin A deficiency (49).

In an economic costing exercise in Nepal the authors identified that the cost of death averted by vitamin A being provided in the country by Female Community Health Volunteers was $327 (50). Quite apart from the social benefits of empowering these poorly educated women, the program was identified as reducing the incidence and severity of diarrheal diseases and measles, which in turn reduces the need for Ministry of Health services, thereby saving the Government of Nepal $1.5 million—which when cost savings have been factored in, had saved the government $1.5 million or annually $167 000 (50). Cost-effectiveness studies undertaken on the national vitamin A distribution programs in Ghana and Zambia found the costs per death averted were $277 and $162 respectively (51, 52).

There are also major costs of micronutrient deficiencies associated with humanitarian crises. Women are typically overrepresented in terms of negative impacts of today’s complex emergencies—roughly 70% of refugees and people displaced inside their own countries by armed conflict are women and children. Wherever crises have resulted in compromised access to food, the threat of acute micronutrient deficiencies rises; if a population is already deficient in vitamins and minerals when an emergency unfolds, the impact is worse than if preexisting conditions had been satisfactory. In Bangladesh, for example, a higher intake of vitamin A was associated with a lower risk of severe malnutrition among children directly affected by floods (53). In Indonesia, although the drought and economic crisis of the late 1990s did not have a significant impact on child anthropometry (weight-for-age), child iron status deteriorated sharply during the crisis and still had not recovered to its precrisis level 5 y later (21). The damage to cognitive development and attained schooling among these children is likely to be long lasting.

Studies on women’s status and childhood nutritional status, although not addressing micronutrient status directly, have concluded that there is good evidence to show that a woman’s status impacts on the nutritional status of her child. Because women with higher status (relative to men) have better nutritional status themselves, they are better cared for and provide higher quality care to their children (35). Across countries, relative resources controlled by women tend to increase the share spent on education (54). Educated girls and women have fewer children, seek medical attention sooner for themselves and their children, and provide better care and nutrition for their children (3).

CONCLUSION

It has been shown that: (1) micronutrient deficiencies lead to poor health outcomes; (2) micronutrient deficiencies are responsible for economic costs at individual, community, and national levels; (3) sex affects health and nutrition outcomes; (4) sex may effect micronutrient deficiencies, at least in some cultures, and that where there is a difference it is usually females who are disadvantaged; and, (5) there are major social and economic costs of micronutrient deficiencies, and consequently, benefits of addressing them. What has not been conclusively shown, although the evidence points that way very strongly is that these costs—both social and economic—are greater for females. Where it has been demonstrated, it has not been well quantified. The usual quantitative approaches used in assessing health and nutrition risk may miss this sort of information, as sex and intra-household relations, social networks and informal sector activities are often not uncovered by conventional statistical methods (34, 43, 54).

The 2003 Human Development report was able to conclude that generally, while some progress had been made, “sex inequality undermines women’s capabilities in education and health” (3). More attention to the context-specific nature of micronutrient deficiencies is called for as a first step toward more reliable prevalence estimations and a more rational basis for targeting public health action. The countries with the worst health and
nutritional conditions, Asia and sub-Saharan Africa, would gain most from the broad public health benefits of better nutrition (30). Because it is increasingly accepted that an integrated approach is required to tackle many vitamin and mineral deficiencies (including dietary diversification, fortification and supplementation integrated into programs to control intestinal parasites and malaria, as well as environmental, sanitation, and political interventions), more attention needs to be paid to the ecological, economic, and cultural factors that influence the local consumption and absorption of nutrients by sex.

To achieve sustainable improvement of the nutritional status of children, women’s status should be improved in all regions, but especially in South Asia, followed by sub-Saharan Africa. However, women’s health must also be improved for their own sake so they are able to lead a productive, healthy, and vital role in their societies, which would, in turn, reap the economic and social benefits. One important way of doing this is to ensure that women and female adolescents and children achieve the various micronutrient goals. Investing in female nutrition through long-term, comprehensive life-course based programs will help break the intergenerational cycle of malnutrition, reduce the cost of micronutrient deficiencies; and have multiple other benefits for women, children, their households, and ultimately for nations.

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Multiple micronutrients in pregnancy and lactation: an overview1–3

Lindsay H Allen

ABSTRACT

This overview of multiple micronutrients during pregnancy and lactation emphasizes 2 relatively neglected issues. The first is that maternal micronutrient status in the periconceptional period, and throughout pregnancy and lactation, should be viewed as a continuum; too often these 3 stages are treated and discussed separately from both a scientific and a public health perspective. Iron and vitamin B-12 are included as examples to stress how status at conception affects maternal, fetal, and infant status and health until the child is weaned. The second issue is that while most attention has been focused on a few micronutrients, for example iron and folate as discussed elsewhere in this Supplement, multiple micronutrient deficiencies occur simultaneously when diets are poor. Some of these deserve more attention as causes of poor pregnancy outcome, including other B vitamin deficiencies that result in homocysteinemia, antioxidants, vitamin D, and iodine. In lactation, maternal status or intake of the B vitamins (except folate), vitamin A, selenium and iodine strongly affect the amount of these nutrients secreted in breast milk. This can result in the infant consuming substantially less than the recommended amounts and further depleting stores that were low at birth. While the optimal mode of meeting recommended micronutrient intakes is an adequate diet, in some situations supplementation is also important. Unfortunately, information is lacking on the optimal formulation of micronutrient supplements for pregnant women, and the need to continue these supplements during lactation is not recognized in many situations where maternal and infant health could benefit. Am J Clin Nutr 2005; 81(suppl): 1206S–12S.

KEY WORDS Pregnancy, lactation, multiple micronutrients, iron deficiency anemia, B-vitamin deficiencies, homocysteine

INTRODUCTION

Adequate maternal micronutrient status is especially critical during pregnancy and lactation. In this overview, we draw attention to micronutrient issues that are sometimes neglected in the context of pregnancy and lactation. One of these is the importance of recognizing the continuum of maternal micronutrient status from the periconceptional period through lactation, and of fetal and infant dependency on adequate maternal status through this time. Another is the fact that multiple micronutrient deficiencies are likely to be present in many situations, some of which have been insufficiently appreciated as contributors to poor pregnancy outcomes and infant development.

The main cause of multiple micronutrient deficiencies is a poor quality diet, often due to an inadequate intake of animal source foods (ASF) especially in developing countries. Women who avoid meat and/or milk in wealthier regions of the world are also at higher risk of micronutrient depletion during pregnancy and lactation. Gene polymorphisms can also cause micronutrient deficiencies through impaired absorption or altered metabolism. This usually results in suboptimal maternal status of single nutrients. One example is folate, where maternal polymorphisms may increase the risk of neural tube defects unless dietary intake of the vitamin is sufficient. In some diets high in unrefined grains and legumes the amount of nutrients consumed may be adequate, but dietary constituents, such as phytates and polyphenols, limit their absorption. Diseases such as malaria, and infection with intestinal parasites, also impair status and alter the metabolism of multiple micronutrients. Finally, in pregnancy and/or lactation, the requirements for most nutrients are higher, increasing the risk of inadequate intake.

Several micronutrient deficiencies are well established to be contributors to abnormal prenatal development and/or pregnancy outcome. These include folate, iron, and iodine deficiencies. Less well recognized for their importance are deficiencies of B-vitamins (and subsequently elevated plasma homocysteine concentrations), vitamin D, and iodine. Additional research is needed to establish adverse effects of poor maternal vitamin A and zinc status in pregnancy. Evidence is accumulating that maternal antioxidant status is important to prevent abnormal pregnancy outcomes. In lactation, maternal status of these same micronutrients (except zinc) affects their concentrations in breast milk. Little attention has been paid to the adverse consequences of micronutrient depletion on maternal health and function during this period.

IRON DEFICIENCY AND ANEMIA

The importance of adequate iron status in pregnancy is emphasized elsewhere in this Supplement. Here, iron is discussed as an example of the need for adequate maternal status of a micronutrient from conception through lactation. Though there is still some controversy concerning the optimal stage of pregnancy at which to begin iron supplementation, several studies have now shown that iron stores at conception are a strong predictor of maternal iron status and risk of anemia in later pregnancy (1, 2).

1 From the US Department of Agriculture, ARS–Western Human Nutrition Research Center, and Department of Nutrition, University of California, Davis, CA.
3 Address reprint requests to LH Allen, Department of Nutrition, University of California, Davis, CA 95616. E-mail: lhallen@ucdavis.edu.
Maternal iron deficiency early in pregnancy has been hypothesized to predict the risk of preterm delivery, based on the fact that risk of conditions such as preeclampsia and premature delivery can be predicted based on hormone concentrations established by mid-pregnancy (3, 4). Also it is very difficult to replenish depleted iron stores once pregnancy is in progress. For example, Swedish women who were not taking iron supplements had virtually no iron remaining in their bone marrow by late pregnancy, compared with 35% of those who consumed 100 mg of iron daily from 16 wk of gestation (5).

One reviewer of the evidence concluded a few years ago that there was insufficient evidence to establish causality between maternal iron status or anemia, and low birth weight or preterm delivery, due to the inadequate number of adequately designed investigations (6). One severe limitation to adequately designing iron intervention studies in pregnancy has been the exclusion of women with anemia at baseline, and/or lack of a placebo group, due to ethical concerns. Using an innovative approach to limit this problem, Cogswell et al conducted an intervention in Cleveland that provided 30 mg iron daily from <20 wk to 28 wk of gestation. A placebo group was included because women found to have a hemoglobin concentration <100 g/L or ferritin <20 µg/L at 28 wk or 38 wk of gestation were supplemented with iron (7). Iron supplementation from enrollment through 28 wk of gestation did not affect the prevalence of anemia but increased birth weight by 206 g and lowered the incidence of low birth weight infants from 17% to 4%. Preterm delivery incidence was not affected, perhaps because supplementation was not started early enough in pregnancy (4). This intervention should be replicated in other populations.

While fetal iron status has been assumed to be relatively independent of maternal status during pregnancy, it is becoming clear that maternal iron status strongly affects the iron stores of the infant at birth. In Indonesia, De Pee et al observed that, compared with a normal birth weight infant born to a mother without anemia, a similar infant born to an anemic mother had a 1.8 times greater risk of developing anemia by 3 to 5 mo of age (8). The highest prevalence of anemia at 3 to 5 mo occurred in low birth weight infants whose mothers were anemic during pregnancy, with an odds ratio of 3.7 compared with normal birth weight infants born to nonanemic mothers. A substantial number of both observational (9, 10) and iron intervention (5, 11, 12) trials support this relation between infant and maternal iron status.

Another neglected problem is that women are often iron-depleted postpartum. Even for women in the United States who were enrolled in the Women, Infants, and Children (WIC) program, postpartum anemia occurred in 27% overall and in 48% of non-Hispanic blacks. The risk of postpartum anemia was greatest in those who were anemic in pregnancy; 49% of women who were anemic in trimester 3 developed anemia postpartum compared with 21% who were nonanemic (13). Anemia postpartum is associated with increased risk of postpartum depression (14).

These examples demonstrate that steps should be taken to ensure that maternal iron status is adequate early in pregnancy, throughout pregnancy and during the postpartum period. Inadequate attention has been paid to the problem of risk of iron deficiency in infants born to iron depleted mothers and to maternal iron status postpartum.

B-VITAMIN DEFICIENCIES AND HOMOCYSTEINEMIA IN PREGNANCY

The importance of adequate maternal folate status in the periconceptional period is discussed elsewhere in this Supplement. There is increasing interest in the fact that homocysteineemia is associated with a greater risk of adverse pregnancy outcomes. Deficiencies of folate, riboflavin, vitamin B-6, or vitamin B-12 lead to elevated plasma homocysteine (Hcy) concentrations. In a retrospective study of 5883 Norwegian women and their 14 492 infants there was a strong association between current Hcy concentrations and previous adverse pregnancy outcomes (15). Women in the highest versus the lowest quartile of plasma Hcy had a history of substantially more placental abruption, still-births, very low birth weight and preterm deliveries, preeclampsia, and club-foot and neural tube defects in their offspring. In a group of 93 Spanish women and their infants, fetal cord plasma Hcy concentration and birth weight were related to maternal plasma Hcy concentration before conception and throughout pregnancy (16). Women in the highest tertile of plasma Hcy at 2 mo of pregnancy had a 3.26 times greater risk of their infant being born in the lowest weight tertile. These odds increased to a 4 times greater risk for women in the highest Hcy tertile at labor, such that their infants weighed 228 g less than those born to mothers in the low and medium Hcy tertiles.

Folic acid supplementation of women in Spain significantly enhanced the physiologic reduction in plasma Hcy that occurs during pregnancy, when the supplements were provided during the 2nd and 3rd trimesters (17). Plasma Hcy appears to respond to supplementation with folic acid up to about 500 to 600 µg of folic acid per day. In addition to poor B vitamin status, other risk factors for elevated plasma Hcy include a high intake of coffee, smoking, and no use of vitamin supplements during pregnancy (15).

More attention needs to be paid to vitamin B-12 status of women during pregnancy and lactation. It has become apparent that there is a high global prevalence of low plasma vitamin B-12 concentrations in infants, children, and adults. For example, a recent review of available data from Latin America revealed that at least 40% of individuals in all age groups studied had low plasma vitamin B-12 (18). Other reports of a high prevalence of vitamin B-12 deficiency include those from Indian adults (19), Kenyan schoolers (20), and pregnant women in Nepal (21). The cause of these low plasma vitamin B-12 concentrations is most likely low dietary intake of the vitamin. While it is commonly believed that only strict vegetarians (vegans) are at substantial risk of developing vitamin B-12 deficiency, several studies have revealed that even lacto-ovo vegetarians (22), or individuals who consume low amounts of meat (23), have lower plasma vitamin B-12 and are at greater risk of vitamin B-12 deficiency compared with omnivores. Apart from studies on folate, there are few data on the relation between the B vitamin status of pregnant women and vitamin B-12 concentrations. In a group of 93 Spanish women and their infants there was a strong association between current Hcy concentrations and adverse pregnancy outcomes. Urban Nepali pregnant women had a 65% prevalence of low plasma B-12, with deficiency associated with higher plasma Hcy and a doubling of risk of preeclampsia and preterm delivery (21). In poor rural pregnant Nepali women the prevalence of low erythrocyte riboflavin concentrations was 89%, of low plasma vitamin B-6, 80%, and of low plasma vitamin B-12, 49%. Riboflavin supplementation alone significantly reduced plasma Hcy, but interventions were not tested with the other B vitamins (24). Low maternal...
plasma vitamin B-12 has been reported to be associated with increased risk of very early recurrent abortion (25), neural tube defects (26), and spina bifida (27).

In summary, poor maternal B-vitamin status may be a major global cause of homocysteinemia and poor pregnancy outcomes. It has not been established how homocysteinemia affects pregnancy outcome adversely, but proposed mechanisms include: Hcy increases oxygen free radical concentrations and reduces nitrous oxide concentrations, leading to endothelial dysfunction (28); Hcy causes oxidative stress and subsequent placental ischemia; Hcy causes an inflammatory response that is cytotoxic to endothelial cells; B-vitamin deficiencies lead to hypomethylation of DNA and altered gene expression, and Hcy induces apoptosis of endothelial cells (29); birth defects may be caused by Hcy interference with the N-methyl-D-aspartate receptor system (30); or Hcy is thrombogenic (31).

OTHER MICRONUTRIENTS IN PREGNANCY

The potential adverse effect of poor vitamin A status on pregnancy outcome was demonstrated in an intervention study in a region in Nepal with endemic vitamin A deficiency (32). Supplementation of these women with approximately their recommended daily intake of vitamin A reduced maternal mortality by 40%. Supplementation with β-carotene reduced mortality by 49%. The apparent cause of the reduced mortality risk was less susceptibility to infection. This trial is currently being repeated in Bangladesh and Ghana. An additional advantage of vitamin A supplementation of pregnant women is that it can increase hemoglobin concentrations, by about 10 g/L in marginally deficient populations (26). The upper limit for retinol supplements is 3000 IU per day based on the potential for higher doses to cause teratogenic effects. β-carotene supplements have not been reported to increase risk of birth defects.

There has been much debate about the efficacy of zinc supplementation in pregnancy. Maternal zinc status was associated with birth weight in 17/41 observation studies. In 6/12 trials there were some benefits (reduction in premature delivery in 3 trials; higher birth weight in 3 trials; reduction in hypertension in 1 trial). Zinc supplementation of Peruvian women did not affect fetal dimensions, birth weight, or the incidence of preterm delivery (33). However, it did reduce fetal heart rate, and increase heart rate accelerations and variability, and fetal movements, which was interpreted to indicate better fetal development (34). In Bangladesh, maternal supplementation in pregnancy had no effect on birth weight, but reduced morbidity of low birth weight infants in the first 6 mo of life (35).

Vitamin D status of pregnant women should be of greater concern even in industrialized countries such as the United States. In the National Health and Nutrition Examination Survey (NHANES) III (1988–1994), 42% of African American women and 4% of Caucasian–non-Hispanic women in 7 states had low plasma concentrations of 25-hydroxyvitamin D (36). Low values were predicted by less intake of fortified breakfast cereals and milk, season, and no use of vitamin D supplements. Vitamin D deficiency is becoming increasingly common in adolescents. Women whose clothing covers a high proportion of their skin, or whose skin is highly pigmented, are at greater risk of vitamin D deficiency. Even moderately low plasma 25-hydroxyvitamin D concentrations observed in Parisian women at the end of winter were associated with poor fetal and infant skeletal growth and mineralization, and poor infant tooth mineralization. These biochemical and clinical signs of vitamin D deficiency were prevented by maternal supplementation with the vitamin (37).

Little attention has been paid to the potentially important issue of antioxidant nutrient status in pregnancy. Oxidative stress caused by free radicals has been implicated in many studies of the etiology of preeclampsia (38). Because ascorbic acid and vitamin E inhibit free radical formation, a double-blind randomized trial was conducted in 283 women who had either a previous history of pregnancy complications or an abnormal ultrasound (39). The supplement provided 1000 mg ascorbic acid and 400 IU vitamin E daily from week 16–22 of pregnancy, and resulted in a 76% reduction in preeclampsia, and a 21% reduction in indicators of endothelial activation and placental dysfunction.

Because even mild maternal iodine deficiency can affect fetal mental development adversely, adequate iodine status in pregnancy is critically important (40). Universal salt iodization has greatly reduced the prevalence of iodine deficiency worldwide but for various reasons, intakes of iodine are often still inadequate. Pregnancy tends to increase the appearance of clinical and biochemical symptoms of iodine deficiency in women with marginal iodine status, resulting in abnormal thyroid hormone concentrations in countries such as Belgium (40). Indeed, a recent review of the need for iodine supplementation found that the majority of women in Europe are iodine deficient during pregnancy but that many prenatal micronutrient supplements did not include iodine (41). In a recent national survey in the United States, 7% of pregnant women and 15% of women of child-bearing age had low urinary iodine (42). Although signs of iodine deficiency are rare in the United States, it may be important for pregnant women who do not purchase iodized salt to ensure that their multiple micronutrient supplements contain iodine, but there is little specific information on this question.

MICRONUTRIENTS IN LACTATION

Exclusive breast-feeding is now recommended by all international agencies for the first 6 mo of life, because of the documented benefits of this feeding strategy for infant health and survival. While agencies such as the WHO emphasize the importance of paying attention to the nutritional status of the lactating woman (43), this is rarely done in practice. Maternal micronutrient deficiencies during lactation can cause a major reduction in the concentration of some of these nutrients in breast milk, with subsequent infant depletion (44). Based on a categorization of the relation between maternal status or intake of each nutrient and its effect on the nutrient concentration in breast milk, “priority” nutrients for lactating women include thiamin, riboflavin, vitamins B-6 and B-12, vitamin A, and iodine (45). This based on the fact that low maternal intake or stores reduces the amount of these nutrients in breast milk, and maternal supplementation can reverse this problem.

Our current understanding of vitamin B-12 deficiency in the perinatal period illustrates the continuum of micronutrient deficiency depletion. Recent data show a strong association between maternal and infant plasma vitamin B-12 concentrations at delivery indicating that maternal B12 status in pregnancy affects infant status at birth (46). Poor maternal intake in lactation can lead to further infant depletion due to the low secretion of vitamin B-12 in breast milk. For example, 62% of rural Mexican women had low concentrations of vitamin B-12 in breast milk at 6 to 8 mo
of lactation (47). In peri-urban Guatemala City, 31% of breast milk samples were low in vitamin B-12 at 3 mo postpartum (48), and 62% of infants in the same community had deficient or low vitamin B-12 concentrations in plasma at age 7 to 12 mo (49). The vitamin B-12 status of these infants was inversely related to the amount of breast milk that they consumed, because those who consumed less breast milk consumed more cow’s milk, which contains about 10 times more vitamin B-12 than even the breast milk of well-nourished women. Less information is available on other B-vitamins in lactation but evidence from various studies indicates that deficiencies are prevalent in some regions of the world resulting in low breast milk concentrations and inadequate infant intakes (44). Breast milk iodine concentrations are very sensitive to maternal status (50) although little is known about how improving maternal status during lactation might improve infant development; providing a high dose of iodine directly to Indonesian infants aged 6 wk reduced mortality substantially during the subsequent 4 mo (51).

The situation with vitamin A status in lactation is slightly different. Infants’ liver stores of vitamin A at birth are very small even in well-nourished populations, so they are greatly dependent on dietary intake of the vitamin. Breast milk is a good source of vitamin A and clinical vitamin A deficiency is rare in breastfed infants during their first year of life, even in poor populations. The normal concentration of retinol in breast milk is about 485 μg/L but in areas of endemic vitamin A deficiency the concentrations can fall below 300 μg/L, and many infants become depleted. To prevent this situation, high dose supplements of vitamin A are provided to mothers postpartum and to infants as part of the Expanded Program on Immunization (EPI) (52). Maintaining an adequate daily supply of vitamin A in breast milk sustains infant vitamin A status better than the sporadic administration of high dose vitamin A supplements, because liver stores of the latter are typically depleted between EPI visits (53).

In contrast to B vitamins, vitamin A, and iodine, the iron content of breast milk is not affected by maternal nutritional status. The iron content of breast milk is insufficient to maintain infant iron stores during the first year of life, but providing maternal iron supplements during lactation cannot rectify this situation. The general recommendation is therefore to provide iron supplements to infants after 6 mo of age, and after 2 mo of age in the event of preterm delivery and/or low birth weight. Concern has been raised about whether iron supplementation of infants and young children increases morbidity and mortality from infections, especially for those who are not iron deficient. Nonanemic infants who were supplemented with iron in either Sweden or Honduras had significantly more diarrheal events than a placebo group (54). Erythropoietic response to iron may be immature in young infants (55), and they may be especially sensitive to free radical formation and oxidative stress induced by iron supplements (56, 57). It may therefore be safer to provide iron to infants in foods, including fortified foods, and only if they are iron deficient or live in areas with a high prevalence of iron deficiency anemia.

In a recent review we summarized the available data on how maternal depletion affects the infant’s intake of micronutrients in breast milk (44). In general, infants will consume only about half of their recommended Adequate Intake (AI) of the “priority” micronutrients if their mother is depleted (Table 1).

**PROVISION OF MICRONUTRIENTS TO PREGNANT AND LACTATING WOMEN**

There are 3 main strategies for increasing maternal intake of multiple micronutrients. The first is to improve dietary quality, which in many situations might require increasing consumption of animal source foods, fruits, and vegetables. A number of studies have reported an association between poor maternal diet and a greater risk of pregnancy complications (58). Also, the relatively few adequately designed intervention studies show that provision of micronutrient-rich foods can improve pregnancy outcome (59, 60). In some situations well-designed nutrition education programs can improve dietary quality and pregnancy outcome (61).

An easier and more common approach is to provide multiple micronutrient supplements to women on their first clinic visit. The efficacy of this approach to improving pregnancy outcome has not been studied adequately. In an observational study of low-income pregnant adolescents in New Jersey, pregnancy outcomes of those who used supplements was compared with those who did not (62). For women who started taking supplements in their first trimester, there was a substantial reduction in preterm,
very preterm, low birth weight and very low birth weight deliveries. If supplements were started in the second trimester, a similar pattern of response was observed, although reduction in complications was somewhat less substantial. In one of the better-known studies of the effects of folic acid supplementation on recurrence of neural tube defects, one arm of the study provided 12 vitamins including folic acid, 4 minerals and 3 trace elements, while the other arm provided only 3 trace elements. The supplement that contained most micronutrients reduced neural tube defects by 90%, and birth defects by 50%, compared with the trace element supplement (63). Women in this group also had less pregnancy complications and morning sickness. When the micronutrients were consumed before pregnancy, menstrual periods were more regular, time to conception was shorter, and the rate of conception was increased by 7%. Two more recent studies compared the benefits of providing multiple micronutrients including iron compared with iron alone. In Mexico (64, 65) and Nepal (66) multiple micronutrient supplements provided 100–150% of the RDA of 15 vitamins and minerals from around 13 wk of gestation. The supplements containing multiple micronutrients were no more effective than iron in improving maternal hemoglobin at 1 mo postpartum, birth weight or gestational age, or (only studied in Nepal) mortality of the infant in the first 6 mo of life. In contrast, multiple micronutrient supplementation of HIV positive pregnant women in Tanzania reduced low birth weight by 44%, preterm delivery by 39%, and IUGR by 43% (67). Although multiple micronutrient supplementation is theoretically preferable to supplementation with iron and folic acid alone, especially in developing countries where multiple deficiencies are prevalent, more data needs to be collected to determine the advantages of different multiple micronutrient formulations for pregnant and lactating women.

CONCLUSION

Even in some industrialized countries there is strong evidence for the benefits of maternal supplementation with iron, folic acid, and vitamin D and at least in Europe, iodine. There is also some evidence that high quality diets and or multiple micronutrient supplements are beneficial for pregnant women even in industrialized countries. The reasons for inconsistent beneficial effects in the multiple micronutrient trials in developing countries is not yet clear. More efficacy trials are needed to test the benefits of neglected nutrients, such as B-vitamins, in both industrialized and developing countries.

Ideally, micronutrient deficiencies should be prevented or treated before a woman becomes pregnant. This will improve fertility and maternal health. To maximize the reduction of birth defects it is important to prevent maternal micronutrient deficiencies in the periconceptional period, but about 50% of pregnancies are unplanned even in the United States and entry to healthcare may be late. Early prevention of B-vitamin deficiencies may also be important to prevent homocysteinemia and iron depletion throughout pregnancy, and to prevent preterm delivery, which is programmed by the middle of gestation. A theoretically reasonable approach to test would be the efficacy of giving multiple micronutrient supplements once or twice a week to women capable of becoming pregnant and then possibly more frequently during pregnancy.

Finally, because micronutrient status of the lactating woman is critical for the secretion of adequate micronutrient concentrations in breast milk, and for the micronutrient status of her infant, more attention should be paid to the micronutrient status of lactating women. Provision of multiple micronutrient supplements and/or food fortification with micronutrients may be advisable for the majority of lactating women in developing countries and industrialized countries—not least to prevent their further depletion through the secretion of large amount of these micronutrients in breast milk.

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MULTIPLE MICRONUTRIENTS IN PREGNANCY AND LACTATION 1211S


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Folic acid supplementation and the occurrence of congenital heart defects, orofacial clefts, multiple births, and miscarriage

Lynn B Bailey and Robert J Berry

ABSTRACT

Key research findings relative to the question of whether maternal use of folic acid before and during pregnancy reduces the chance that offspring will be born with a congenital heart defect or an orofacial cleft are reviewed in this paper. Observational studies in general support an association between maternal use of multivitamins containing folic acid and a reduction in the occurrence of congenital heart defects and orofacial clefts. Results from one randomized controlled trial (RCT) provide the strongest evidence that multivitamins prevent congenital heart defects, but this RCT did not provide evidence that multivitamins prevent orofacial clefts. In addition, most observational and interventional studies are not designed to detect an independent effect from folic acid. Early studies suggested that periconceptional multivitamin use was associated with an increased occurrence of both miscarriages and multiple births, which has resulted in a great deal of controversy about the safety of folic acid use during pregnancy. We also review reports that were designed to answer these questions with more definitive data. When more substantial evidence about the effect of periconceptional folic acid on the occurrence of congenital heart defects and orofacial clefts is reported, we will have additional support for promoting folic acid intervention programs. All women capable of becoming pregnant should continue to consume 400 µg/d of folic acid in addition to a healthy diet as advised. Am J Clin Nutr 2005;81(suppl):1213S–7S.

KEY WORDS Folic acid, heart defects, orofacial clefts, miscarriages, multiple births

INTRODUCTION

Periconceptional consumption of folic acid, or multivitamins containing folic acid, has been shown in intervention studies to reduce the risk for neural tube defects (NTDs) (1–3). The successful translation of these scientific data related to folic acid and NTD risk reduction into public health policy provides the rationale for evaluating evidence for an association between periconceptional folic acid and other common congenital anomalies including heart defects and orofacial clefts. In this paper, key findings related to periconceptional use of multivitamins containing folic acid and a reduced occurrence of both congenital heart defects and orofacial clefts are briefly reviewed.

The controversial claims that periconceptional folic acid use will increase the occurrence of both multiple births (4–6) and miscarriages (7, 8) are of concern to women of reproductive age and their health care providers. Most of these studies analyzed data collected for other purposes which introduced many potential flaws. More recent findings from a large-scale intervention study fail to confirm an increased risk for either multiple births or miscarriages associated with maternal use of folic acid before and during early pregnancy (3).

An RCT testing whether different doses of folic acid or folic acid plus other vitamins reduce the risk for other birth anomalies including heart defects and orofacial clefts is needed before specific public health recommendations are made. The appropriate advice to women capable of becoming pregnant is to follow the current folic acid recommendation designed to reduce the occurrence of NTDs.

CONGENITAL HEART DEFECTS

Heart defects affect 1 in 110 newborns and account for a third or more of infant deaths due to birth defects, more than that for any other congenital anomaly including NTDs (9–12). In the United States alone the number of deaths attributed to heart defects is estimated to be ~6000/y (13). There has been an extensive investigation of potential causes and risk factors for heart defects; however, only a very small percentage of cases can be definitively linked to known etiology (10).

The strongest evidence that multivitamins containing folic acid taken periconceptionally will significantly reduce the risk of congenital heart defects is supported by data from a Hungarian RCT (2) and from 2 population-based case-control (PCC) studies in the United States (14, 15). The Hungarian RCT was designed to evaluate the efficacy of a prenatal multivitamin containing 800 µg of folic acid in reducing the occurrence of NTDs (2). Further analysis of this RCT and subsequent data from Hungary showed that prenatal multivitamins were associated with an impressive overall 50% reduction in risk for a broad range of heart defects (Figure 1) (16, 17). In an Atlanta PCC study, use of periconceptional multivitamins containing folic acid was associated with a 24% reduction in the odds for congenital heart defects in general

Although the observed association was less than that observed in the Hungarian RCT (2, 17), data from the Atlanta PCC study also support the conclusion that periconceptional use of multivitamins significantly reduces the occurrence of congenital heart defects (14). When evaluating the association with specific types of heart defects, the data suggest that the association may be strongest for ventricular septal defects (VSD) and some conotruncal defects (tetralogy of Fallot and D-transposition of the great arteries) (18). In both the Atlanta PCC study and the Hungarian RCT (14, 17), the occurrence of conotruncal defects was reduced (~50%) (Figure 1). Data from a different PCC study (15) also support the conclusion that periconceptional multivitamins are associated with a reduction (30%) in the occurrence of conotruncal defects. In contrast, Werler et al (19) detected no association between 2 types of congenital heart defects (VSD and outflow tract) and periconceptional multivitamin use in a hospital-based case-control study. Also, Scanlon et al (20) detected no association between outflow tract defects and periconceptional multivitamin use in the Baltimore-Washington Infant Study. The majority of evidence supports the conclusion that periconceptional multivitamins containing folic acid may reduce the risk for congenital heart defects.

Which nutrient or combination of nutrients in multivitamins that is responsible for the apparent reduction in congenital heart defects has not been established. No direct evidence that folic acid alone is responsible has been published. However, some indirect evidence does exist. Analysis of a large case-control study (21) evaluated, among women who took drugs that inhibit dihydrofolate reductase, which is required for normal DNA synthesis, whether taking these folic acid antagonists during early pregnancy increased a woman’s risk of having an infant with a congenital heart defect and whether concomitant use of multivitamins influenced this risk. The relative risk associated with the use of the folic acid antagonists when no multivitamin supplement containing folic acid was taken was 7.7 [95% confidence interval (CI), 2.8 to 21.7]. In contrast, when multivitamins were taken in addition to the drugs (trimethoprim, triamterine, and sulfasalazine), the relative risk associated with the use of both the drugs plus supplement was reduced to 1.5 (95% CI, 0.6 to 3.8). These findings indicate that the use of folic acid antagonists in early pregnancy increases the risk of heart defects particularly among the infants of women who did not take multivitamins, but that multivitamins could attenuate this increased risk. These data support the conclusion that folic acid is the active ingredient in multivitamins, that folic acid is essential for normal fetal cardiac development during early embryogenesis, and that periconceptional folic acid use may reduce the risk for congenital cardiac anomalies.

**OROFACIAL CLEFTS**

Orofacial clefts including cleft lip with or without cleft palate (CLP) and cleft palate alone (CP) affect ~1 in 1000 and ~1 in 2500 infants, respectively (22). Studies have resulted in mixed findings related to the protective effect of maternal multivitamins containing folic acid on the occurrence of orofacial clefts. Maternal use of multivitamins was associated with a significant reduction in the occurrence of orofacial clefts in a PCC study based on an analysis of data from the California Birth Defects Monitoring Program (23). In this study, a 50% reduction in the occurrence of CLP and a 27% reduction in the occurrence of CP was associated with maternal use of multivitamins (23). A similar finding for CLP (48%) was reported by Itikala et al (22) among women who used multivitamins during the periconceptional period or who started multivitamin use during the first postconception month. In contrast, no association was observed in a hospital-based case control study in the Boston, Philadelphia, and Toronto areas (24). However, in a subsequent investigation in this same area, Werler et al (19) found significantly lower odds for CP, but not CLP, among mothers who took multivitamins containing folic acid during pregnancy and gave birth to healthy infants compared with case mothers. Recently periconceptional folic acid supplement use was reported to halve the risk of CLP (25). The findings from this investigation support the previously reported 50% reduction in occurrence of CLP with periconceptional folic acid-containing multivitamin use (22, 23).
only folic acid strengthen the direct evidence that the association between maternal use of periconceptional folic acid and a reduction in the occurrence of CLP is true (25).

A significant protection against recurrence of CLP was reported in response to supplementation with multivitamins containing a very high dose of folic acid (10 mg) in a Hungarian study (26). In contrast, no protection against the occurrence of CLP was detected in a cohort study with lower doses (800 μg) of folic acid in a multivitamin supplement (27). In addition, data from the Hungarian RCT do not support the conclusion that periconceptional use of multivitamins containing folic acid may reduce the risk for orofacial clefts (28).

Indirect evidence that the folic acid component of multivitamins taken periconceptionally reduces the risk for orofacial clefts is supported by findings from a large case-control study in which folic acid antagonists were shown to increase the risk for orofacial clefts (21). The relative risk of orofacial clefts in infants whose mothers were exposed to dihydrofolate reductase inhibitors during early pregnancy as compared with infants whose mothers had no such exposure was 2.6 (95% CI, 1.1 to 6.1). In contrast, the relative risk of orofacial clefts associated with the use of dihydrofolate reductase inhibitors with no multivitamins containing folic acid was 4.9 (95% CI, 1.5 to 16.7). These findings support the conclusion that the folic acid component of multivitamins may reduce the risk for orofacial clefts.

**MULTIPLE BIRTHS**

A number of reports have suggested the possibility of a significant increase in the occurrence of multiple births among women who take multivitamins containing folic acid during early pregnancy (4–6). Since multiple births result in more pregnancy complications, are more likely to result in preterm delivery and are associated with an estimated sevenfold increase in mortality compared with singleton pregnancies (29–32), these reports warrant careful evaluation. An analysis of data from an RCT by Czeizel et al indicated a 40% increase in the number of infants resulting from multiple births among women who received multivitamins containing 800 μg folic acid compared with the number of infants resulting from multiple births of women who received only trace elements (4). In this study ovarian stimulating drugs were used by 6.5% of the women from whom 42% of the multiple pregnancies were produced. Among these women the rate of multiple pregnancies was 10.92%, 11 times higher than the rate of 1.03% among women who did not use ovarian stimulation. When women who used ovarian stimulation are excluded, and the appropriate comparison is made, the increased risk estimate is unchanged, but the statistical significance disappears [risk ratio (RR), 1.45; 95% CI, 0.78 to 2.67]. In 1997, Werler et al retrospectively analyzed data on multiple and singleton births collected from 3 separate birth defect programs and reported a nonsignificant 30% to 60% greater prevalence of periconceptional vitamin supplementation among mothers of multiple births (5). Information about supplement use in these studies was collected retrospectively by interview and did not differentiate among types of vitamin supplements used. Only 2 programs could evaluate the occurrence of multiple births among women who delivered normal infants. Among these women, those who took multivitamins any time during pregnancy showed no increase in the rates of twinning when compared with those who did not take multivitamins, both in Atlanta (OR, 0.92; 95% CI, 0.46 to 1.45) and in California (OR, 0.80; 95% CI, 0.22 to 2.82).

Recently, Ericson and colleagues described an increase in the occurrence of dizygotic twin deliveries among women reporting the use of folic acid during early pregnancy in Sweden, compared with the rate of twin births in the entire Swedish population (OR, 2.13; 95% CI, 1.64 to 2.74) (6). The overall rate of reported folic acid consumption in this study was < 1% which is markedly different from the consumption in the United States, where ~40% of women of childbearing age take multivitamins containing folic acid (33). In addition, the authors state that their results were highly confounded by increasing maternal age and the length of involuntary childlessness, which raises questions about whether most of the women who reported folic acid use in this study were also using assisted reproductive technologies.

If the occurrence of multiple births were influenced by consumption of folic acid, the effect might be expected to be greater with increasing folic acid dose; however, this has not been observed. In 1999, Mathews et al analyzed data from the United Kingdom Medical Research Council (MRC) Vitamin Study (1) in which women consumed a multivitamin containing 4000 μg folic acid per day and did not detect a significant difference in the rates of multiple birth between supplemented and unsupplemented women (34).

Most of the reports suggesting that the periconceptional use of multivitamins containing folic acid increased the occurrence of multiple births have focused on the use of folic acid as the purported cause of the increase despite the fact that most women in these studies took folic acid combined with multivitamins, not folic acid alone. For example, the multivitamins taken by women in the Hungarian study (4) contained 10 other components, including 4000–6000 international units (IU) of vitamin A, which has been reported to be associated with an increased rate of twin births in a randomized controlled trial in Nepal (35, 36).

Evaluation of data from a large-scale (~250,000 women) intervention study in China where women took 400 μg/d of folic acid daily before and during early pregnancy to prevent NTDs (3) provides strong evidence that periconceptional folic acid use does not increase the occurrence of multiple births (37). Overall, the rate of multiple births was not different among women who took folic acid daily compared with those who did not (0.59% and 0.65%, respectively). This study has important strengths including the fact that the results were not confounded by other factors that may have increased occurrence of multiple births. For example, other studies have been conducted in populations where the rates of multiple births may have been affected by increasing maternal age and the use of ovarian stimulation or assisted reproductive technologies (4, 6). In the China study, the study population comprised a cohort of young women, most of whom were experiencing their first pregnancy, and in whom the treatment for subfertility was extremely low. For this reason, the young Chinese women represented an excellent group for studying the occurrence of multiple births because it was not necessary to control for the presence of these factors. Another strength of the China study was that precise records of supplement-taking were collected prospectively, before the outcome of pregnancy was known, minimizing the potential for recall bias. Possibly the most important aspect of this study was that unlike others, the women consumed a supplement containing only 400 μg of folic acid. This large population-based study provided strong evidence that consumption of 400 μg of folic acid alone before and
during early pregnancy did not increase a woman’s likelihood of having a multiple birth.

In the United States, the rate of multiple births has been recently evaluated before and following the mandatory introduction of folic acid fortification in 1998. After adjusting for both increasing maternal age and use of assisted reproductive technology, no evidence that multiple births have increased since the start of folic acid fortification was detected (38–40).

MISCARRIAGE

In 1997, Hook and Czeizel analyzed data from the Hungarian RCT (2) and concluded that periconceptional use of a multivitamin containing 800 µg folic acid was associated with a significant 16% increase in miscarriage rates compared with women who received trace elements (7). In addition, these investigators analyzed data from the MRC (1) study including all women assigned periconceptional folic acid treatment (4000 µg) and reported a nonsignificant 15% increase in miscarriage rates (7). Based on an analysis of these data by MRC study researchers including only data from women who became pregnant, there was no association between periconceptional folic acid supplementation and an increase in miscarriage rates (RR, 1.06; 95% CI, 0.79 to 1.43, P = 0.70) (41). More recently, Windham et al (8) reported that data from a prospective study of women in California interviewed during their first trimester of pregnancy supported the conclusion of Hook and Czeizel (7) that periconceptional vitamins increased the occurrence of miscarriage. In this study, a nonsignificant increase in the occurrence of miscarriage among women who took vitamins during the prenatal period (RR, 1.14; 95% CI, 0.96 to 1.35) was observed and attributed to periconceptional folic acid use although the supplements taken were a mixture of vitamins not folic acid alone (8). Other investigators, however, disagreed that this report provided evidence that folic acid increased the occurrence of miscarriage (42).

Data from a large-scale folic-acid intervention study conducted in China provided strong evidence that periconceptional folic acid use does not increase miscarriage rates (43). In this study, the miscarriage rate was 9.0% for women who took folic acid alone (400 µg) and 9.3% for women who did not take folic acid during early pregnancy (RR, 0.97; 95% CI, 0.84 to 1.12). The strengths of this study include the fact that the investigation was larger than all of the previous studies combined (6, 8); the study population was confined to women who were registered in the program before they became pregnant for the first time (avoiding confounding by previous miscarriages); supplement-taking data were obtained during pregnancy (avoiding recall bias); and supplements contained folic acid alone.

The association between folate status and the occurrence of miscarriage was further evaluated in a recent large PCC study in Sweden (44). Cases were women who had spontaneously aborted a fetus with a gestational age of 6–12 wk and controls were women matched for gestational age of the fetus. Women with low plasma folate concentrations (≤4.9 nmol/L) were more likely to have had a miscarriage than women with plasma folate concentrations between 5.0 and 8.9 nmol/L. The occurrence of miscarriage was not increased in women with higher plasma folate concentrations (≥14.0 nmol/L) relative to women with plasma folate concentrations between 5.0 and 8.9 nmol/L. Supplement use was not associated with an increased occurrence of miscarriage. A major strength of this study was the adjustment for confounders (eg, maternal age, cigarette smoking, previous miscarriages) providing data that further supports the conclusion that a folate deficiency significantly increases the occurrence of miscarriage as previous studies had suggested (45–47).

APPLICATION AND FUTURE DIRECTION

Research findings to date suggest that periconceptional use of multivitamins containing folic acid is associated with a reduction in the occurrence of congenital heart defects and a possible reduced occurrence of orofacial clefts, although the data are less convincing for orofacial clefts. Efforts to draw definitive conclusions from these studies are hampered by the difficulties in interpreting and drawing conclusions due to heterogeneity between studies including factors such as classification of the types of defects and variation in exposure to numerous environmental factors, including maternal use of multivitamins containing folic acid (11, 18). The evolution of science related to folic acid and NTD risk reduction required the careful evaluation of similar types of complex and often conflicting data (48). Resolution of the NTD/folic acid controversy was a direct result of RCTs, which highlights the need for similar large well-designed studies to specifically address whether maternal periconceptional use of other vitamins or higher doses of folic acid will reduce the occurrence of other congenital anomalies, and, if so, whether the protective effect of folic acid is limited to specific types of defects. Collaborative multidisciplinary research endeavors that address the complexities of these research issues are needed before definitive public health recommendations can be implemented (11).

The appropriate medical advice is that all women capable of becoming pregnant should consume 400 µg/d of synthetic folic acid in addition to a healthy diet (49). Although NTD risk reduction is the goal of this existing public health recommendation, if future research confirms that periconceptional folic acid supplementation also reduces the risk for other birth defects, this would augment the benefit to a proven effective way to prevent infant mortality and disability (18). It is reassuring that data do not support the reports that folic acid supplements increase the occurrence of miscarriage or multiple births because women of childbearing age in many countries are advised to take folic acid supplements daily to prevent NTDs. Women of reproductive age and their health care providers can support the periconceptional folic acid supplementation policy without concern that this practice will increase the occurrence of multiple births or miscarriages.

The authors have no conflict of interest.

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FOLIC ACID AND OCCURRENCE OF SELECT BIRTH OUTCOMES 1217S


Iron status during pregnancy: setting the stage for mother and infant1–4

Theresa O Scholl

ABSTRACT
Supplementation with iron is generally recommended during pregnancy to meet the iron needs of both mother and fetus. When detected early in pregnancy, iron deficiency anemia (IDA) is associated with a > 2-fold increase in the risk of preterm delivery. Maternal anemia when diagnosed before midpregnancy is also associated with an increased risk of preterm birth. Results of recent randomized clinical trials in the United States and in Nepal that involved early supplementation with iron showed some reduction in risk of low birth weight or preterm low birth weight, but not preterm delivery. During the 3rd trimester, maternal anemia usually is not associated with increased risk of adverse pregnancy outcomes and may be an indicator of an expanded maternal plasma volume. High levels of hemoglobin, hematocrit, and ferritin are associated with an increased risk of fetal growth restriction, preterm delivery, and preeclampsia. While iron supplementation increases maternal iron status and stores, factors that underlie adverse pregnancy outcome are considered to result in this association, not iron supplements. On the other hand, iron supplements and increased iron stores have recently been linked to maternal complications (eg, gestational diabetes) and increased oxidative stress during pregnancy. Consequently, while iron supplementation may improve pregnancy outcome when the mother is iron deficient it is also possible that prophylactic supplementation may increase risk when the mother does not have iron deficiency or IDA. Anemia and IDA are not synonymous, even among low-income minority women in their reproductive years. Am J Clin Nutr 2005;81(suppl):1218S–22S.

KEY WORDS Anemia, iron deficiency, ferritin, oxidative stress, preterm delivery, low birth weight, gestational diabetes, iron, supplementation, pregnancy

INTRODUCTION
Anemia, as determined by low hemoglobin or hematocrit, is common among women in their reproductive years in particular if the women are poor, pregnant, and members of an ethnic minority. Until recently, it was assumed that anemia during pregnancy had few untoward sequelae. During the past few years, the relation between anemia early in pregnancy and an increased risk of preterm delivery has been suggested. Likewise, the relation of adverse pregnancy outcomes with high hemoglobin and increased iron stores has been documented. However, the risks and benefits of prophylactic iron supplementation in pregnant women who are not iron deficient remains a source of controversy.

PREVALENCE AND ETIOLOGY OF ANEMIA AND IRON DEFICIENCY IN WOMEN
Iron deficiency is the most commonly recognized nutritional deficit in either the developed or the developing world. During their reproductive years women are at risk of iron deficiency due to blood loss from menstruation, in particular that 10% who suffer heavy losses (>80 mL/mo). Contraceptive practice also plays a part—the intrauterine devices increases menstrual blood loss by 30%–50% while oral contraceptives have the opposite effect. Pregnancy is another factor. During pregnancy there is a significant increase in the amount of iron required to increase the red cell mass, expand the plasma volume and to allow for the growth of the fetal-placental unit. Finally, there is diet. Women in their reproductive years often have a dietary iron intake that is too low to offset losses from menstruation and the increased iron requirement for reproduction (1). Consequently, the overall prevalence of iron deficiency in non-pregnant women of reproductive age in the United States, 9%–11%, is higher than at other ages apart from infancy. The prevalence of IDA in the same age group is 2%–5%. Prevalence of iron deficiency and IDA is increased 2-fold or more for those women who are minorities, below the poverty level or with < 12 y of education. Risk is also increased with parity—nearly 3-fold higher for women with 2–3 children and nearly 4-fold greater for women with 4 or more children, thus implicating pregnancy (2).

It is estimated that < 50% of women do not have adequate iron stores for pregnancy (1, 3). Because the iron required for pregnancy (3–4 mg/d) is substantial, risk of iron deficiency and IDA should increase with gestation. However, the prevalence of anemia and IDA in pregnant women from the United States is not well defined but must be substantial, particularly among the poor. During pregnancy, anemia increases > 4-fold from the 1st to the 3rd trimester in the low-income women monitored as part of pregnancy nutritional surveillance by the CDC (3). In the Camden Study where the cohort is mostly minority, current data (2000–2004) suggest that the prevalence of anemia increases >

1 From the Department of Obstetrics and Gynecology, The University of Medicine and Dentistry of New Jersey - SOM, Stratford, New Jersey 08084.
3 Supported by HD18269, HD38329, and ES07437 from the National Institutes of Health.
4 Address reprint requests and correspondence to Theresa O Scholl, UMDNJ-SOM, Department of Ob/Gyn, Science Center, Suite 390, Stratford, NJ 08104. E-mail: scholl@umdnj.edu.
6-fold from 6.7% (1st trimester) to 27.3% (2nd trimester) to 45.6% in the 3rd trimester. Only a fraction of anemic women in Camden have iron deficiency anemia. Based on low hemoglobin for gestation by CDC criteria plus low ferritin (<12), iron deficiency anemia in Camden gravidas is lower—1.8% in 1st trimester, to 8.2% in 2nd trimester, and 27.4% in 3rd trimester (Figure 1). Thus, anemia and IDA are not synonymous, even among low-income minority women in their reproductive years.

Anemia has been called a “sickness index” for the body (4). Apart from iron deficiency, the most frequent reason, and the physiologic anemia of pregnancy (both discussed below) causes include hemoglobinopathies like thalassemia, deficiencies of folate/B12, and the anemia of chronic disease, which ranks second to iron deficiency in prevalence. This anemia develops as part of a host response to a wide range of disorders that also involve the red cell. While anemia of chronic disease is often associated with an underlying condition such as cancer or cardiovascular disease or when an infectious or inflammatory process is chronic, it can also develop when the infection or inflammation is acute. Its diagnosis is one of exclusion (4).

PREGNANCY OUTCOME WITH MATERNAL ANEMIA DETECTED EARLY IN PREGNANCY

Some of the increase in anemia and iron deficiency anemia with gestation is an artifact of the normal physiologic changes of pregnancy (5). Although the maternal red-cell mass and plasma volume both increase during gestation, they do not do so simultaneously. Hemoglobin and hematocrit decline throughout the 1st and 2nd trimesters, reach their lowest point late in the second to early in the 3rd trimester and then rise again nearer to term (6). In late pregnancy it is difficult to distinguish physiologic anemia from iron deficiency anemia (5, 7). It is thus becoming clear that the best time to detect any risk associated with maternal anemia may be early in pregnancy.

We originally studied this issue in Camden by separating anemia at entry to prenatal care and week 28 into iron deficiency anemia and anemia from causes other than iron deficiency (5, 8). Early in pregnancy there were clear differences in mean corpuscular volume (MCV) and diet in women with and without IDA that either were not present or were greatly diminished during the 3rd trimester. At entry, women with iron deficiency anemia had an MCV that was significantly lower (6.5 femtoliters) than other women. During the 3rd trimester the MCV of women with IDA was close to the mean of the other women. At entry, women with IDA had a significantly lower energy intake (500 Kcal/d less) than the others and, iron intake from diet was also significantly less (5 mg less) because of the difference in energy. During the 3rd trimester women with IDA showed little difference in the intake of energy or of iron. At entry, both iron deficiency anemia and anemia from other causes were associated with an increased risks of inadequate weight gain for gestation. For women with IDA, risk was increased 2-fold while for women with other anemias risk was increased by about 50%. In the 3rd trimester, IDA remained associated with a 2-fold risk of an inadequate weight gain for gestation whereas risk was not increased for women with other anemias. IDA at entry was associated with greater than 2-fold increases in the risks of low birth weight and preterm delivery, while anemia stemming from other causes was associated with a only a small increase in risk that was not significant. In the 3rd trimester, risk of preterm delivery was reduced for women with IDA and was not an increased risk for women with other anemias (5, 8).

Scanlon and colleagues recently confirmed the relation between early anemia (based on hemoglobin alone) and preterm delivery with retrospective data from nearly 250,000 low-income gravidas who attended WIC clinics in eleven states (9). Preterm delivery was increased for women with anemia during the 1st or 2nd trimester and risk depended on the severity of the hemoglobin deficit. For women with moderate to severe anemia (equivalent to 95 g/L at week 12), risk was approximately doubled, for women with mild anemia, risk of preterm delivery was increased between 10%–40%. During the 3rd trimester the association reversed—anemic women had a 12%–25% reduction in the risk of preterm birth. Maternal anemia was not associated with any increase in the risk of small for gestation births.

Data from Shanghai also suggested an effect of maternal anemia on preterm delivery that was the most detectable during the 1st trimester, before maternal plasma volume expanded (10). All gravidas were Chinese and showed little variation in parity, smoking, or utilization of prenatal care. Rates of preterm delivery and low birth weight, but not small for gestation births, were increased for women who had anemia early in pregnancy. Risk of preterm delivery and low birth weight were increased >2-fold in moderately anemic women (90–99 g/L) and >3-fold in those who were severely anemic (<90 g/L) during the 1st trimester. At midpregnancy and late in the 3rd trimester, the influence of maternal anemia on pregnancy outcome was markedly attenuated but not reversed. Thus, whether or not maternal anemia increases risk of poor pregnancy outcomes may depend on when in pregnancy the anemia was measured. Several studies have reported reduced risks of preterm delivery or low birth weight or no association between anemia and preterm birth when the relation was studied during the 3rd trimester (11–12).

POTENTIAL MECHANISMS FOR ADVERSE OUTCOMES

If only the women who developed iron deficiency anemia before or early in pregnancy were at increased risk of delivering preterm this might mean that a mechanism that involves iron could be integral to the outcome of pregnancy. Allen (13) suggested 3 potential mechanisms whereby maternal IDA might give rise to preterm delivery: hypoxia, oxidative stress, and infection. Chronic hypoxia from anemia could initiate a stress response, followed by the release of CRH by the placenta, the increased production of cortisol by the fetus, and an early delivery. Increased oxidative stress in iron deficient women that was...
not offset by endogenous or dietary antioxidants could damage the maternal-fetal unit and result in preterm delivery. With reduced immune function and increased risk of infection among iron deficient women, there would be an increased production of cytokines, secretion of CRH, and production of prostaglandin, increasing risk of a preterm birth.

MATERNAL ANEMIA: RANDOMIZED TRIALS OF IRON SUPPLEMENTS

Because data on maternal anemia are from observational studies, it is not certain if the effect of anemia on pregnancy outcome is causal and could be prevented by supplementation with iron. Observational data on anemia imply that iron supplementation should be started early in pregnancy, if not before, to prevent preterm delivery. If this is true, then iron supplementation started after midpregnancy, the usual time for most women, is unlikely to reduce risk. A novel clinical trial was conducted in 275 pregnant women, all WIC participants, none anemic, who were enrolled at entry to care in double blind and randomized trial with supplemental iron (30 mg/d as ferrous sulfate) or placebo until week 28 gestation (14). All women in the trial were enrolled before week 20, and the average gestation at entry to the study was 10.75 ± 3.8 wk gestation. Cut-points, which rendered women ineligible for the trial, were hemoglobin < 110g/L and serum ferritin < 20 μg/L. At weeks 28 and 38, women who were not anemic or iron deficient continued on either the iron or placebo arm. At those points women with serum ferritin < 12 μg/L received 60 mg iron/d and those with ferritin between 12 and < 20 μg/L received 30 mg iron/d, regardless of initial assignment.

Prophylactic iron supplementation from entry to week 28 did not increase maternal serum ferritin or hemoglobin, reduce risk of maternal anemia, or reduce any other measures of maternal iron status in iron supplemented women compared with controls. However, after adjustment was made for 2 factors that differed initially between the groups (pregravid weight and serum ferritin concentration) the proportions with absent iron stores (ferritin < 12 μg/L) and with IDA (Hgb < 110 g/L, ferritin < 12 μg/L) at week 28 were significantly lower among the iron supplemented. Supplemental women had significantly longer gestation durations (+ 0.6 wk), and increased infant birth weight (+ 206 g) than those who were not supplemented. They also showed 4-fold reductions in risk of infant low birth weight and preterm low birth weight. Risk of preterm delivery was not reduced by supplementation but had been reckoned solely from the mother’s last menstrual period (LMP) based on her recall. Failure to confirm or modify the mother’s LMP by ultrasound would introduce an unknown amount of error into an estimate of preterm birth.

Another cluster-randomized study with early supplementation arrived at a similar, but not identical result. Christian et al (15) randomized women residing in geographic sectors of rural Nepal to one of 5 treatment arms. From early pregnancy women received either vitamin A (1000 µg retinol equivalents) alone (control), vitamin A plus folic acid (400 µg), vitamin A plus folic acid plus iron (30 mg). The other 2 arms had added zinc (30 mg) or multiple micronutrients in addition to the Vitamin A. In comparison to controls, gravidas receiving folate showed no reduction in the risk of low birth weight, whereas those receiving iron plus folate increased birth weight by 37 g and showed a reduction of 14% in risk of low birth weight.

PREGNANCY OUTCOME WITH INCREASED IRON STATUS AND STORES

Randomized trials of iron prophylaxis during pregnancy have demonstrated positive effects on reducing low hemoglobin and hematocrit, and increasing serum ferritin, serum iron and other measures, including bone marrow iron (16–17). A recent study of iron containing supplement utilization from NHANES, 1988–94 showed that 72% of pregnant and 69% of lactating women used iron supplements during the month before they were surveyed. However, median consumption of supplemental iron was in excess of the tolerable upper limit of 45 mg/d in pregnant (58 mg/d) and lactating women (57 mg/d) (18). Overall, < 15% of reproductive age women, pregnant and nonpregnant alike, who took iron supplements, had or were being treated for anemia within the past 3 mo. Thus, there is a potential concern that some women who are not anemic may be taking large doses of supplemental iron during pregnancy. It has been suggested that such use may build up the mother’s iron stores and increase blood viscosity so that utero-placental blood flow is impaired or that the excess iron intake could cause other toxic reactions (19).

In addition to their work on anemia, Scanlon and colleagues considered high levels of hemoglobin during the 1st and 2nd trimesters (9). They found that high hemoglobin was associated with an increased risk (5%–79%) of small for gestational age (SGA) births, but not with preterm delivery. Levels that were 1 SD unit or more above the mean marked the threshold for increased risk and were equivalent to 131 g/L at week 12 and 126 g/L at week 18. Likewise, Zhou et al (10) examined high hematocrit along with anemia. During the 1st trimester women with hemoglobin levels exceeding 130g/L showed no increase in the risk of SGA births but had a > 2-fold increases in preterm delivery and infant low birth weight. There were few such women and increased risks were usually not statistically significant. Failure of hemoglobin to fall below 105 g/L was associated with increased risk of poor outcome in a multiethnic sample of gravidas from England (20). In the stratum of women whose lowest hemoglobin was between 126–135 g/L, there was a greater than 2-fold increase in preterm delivery and low birth weight and at the highest level, when hemoglobin remained above 145 g/L, there was a > 7-fold increase in risk of low birth weight and 5-fold increases in risk of preterm delivery.

Hemminiki and Rimpela carried out a clinical trial of selective versus routine iron supplementation in 2912 Finnish women (21–23) to determine whether routine supplementation with iron (100 mg elemental iron from at least 16 wk gestation to delivery) in nonanemic women increased risk of high maternal hemoglobin and poor fetal growth. Women randomized to the selective group received iron supplements only when hematocrit fell below 30% or hemoglobin below 100 g/L on 2 consecutive visits after week 33. In comparison to selective supplementation, routine supplementation with iron halted the decline in hematocrit by week 20 and did not alter infant birth weight, whereas gestation duration was increased significantly (+ 0.2 wk). Interestingly, in both routine and nonroutine groups, a high hematocrit was negatively correlated with birth weight and placental weight; this correlation was first detected during the 1st trimester (23). A recent study from the Netherlands, wherein a cohort of 240 women was monitored from before conception to delivery, underscores this point. Gravidas with an early pregnancy fetal loss had a less profound decline in hematocrit from before conception
to 10 wk postLMP (24). Thus, factors that underlie an adverse pregnancy outcome (poor plasma volume expansion, increased blood viscosity) may give rise to high maternal hemoglobin rather than use of iron supplements.

Iron stores that are elevated for pregnancy are associated with preterm delivery, preeclampsia and gestational diabetes mellitus. Women with ferritin levels that are elevated for the 3rd trimester of pregnancy (>41 ng/mL) have a greatly increased risk of preterm and very preterm delivery that has been attributed to intra-uterine infection (25, 26). Another plausible mechanism for high ferritin levels is failure of the maternal plasma volume to expand. In Camden, increased IDA and lower levels of folate were found in women who went on to have high 3rd trimester ferritin. In the 3rd trimester the situation reversed, thus implicating plasma volume expansion (26). Ferritin production also is increased with infection and inflammation as part of the acute phase response. In the presence of infection, macrophages produce inflammatory cytokines that generate reactive oxygen species, releasing free iron from ferritin (27).

IRON, MATERNAL DIABETES, AND OXIDATIVE STRESS

Iron supplementation during pregnancy increases maternal iron status during pregnancy including hemoglobin, serum iron, MCV, transferrin saturation, and serum ferritin. Reactive oxygen species are products of oxygen. When brought into contact with a transition metal that is capable of changing valence, such as iron, (Fe$^{2+}$ → Fe$^{3+}$) a very reactive free radical, the hydroxyl radical is formed from oxygen via the Fenton Reaction. These free radicals have the potential to damage cells, organs, and tissues in the body (28). Oxidative stress over time is now thought to be a component of the processes of aging, cancer, and the development of cardiovascular disease. Iron overload and the associated oxidative stress contribute to the pathogenesis and increase risk of type 2 diabetes and other disorders. In iron overload, the accumulation interferes with the extraction, synthesis and secretion of insulin (29). It is difficult for reproductive age women to become iron overloaded because of blood loss with menstruation. However, moderately elevated iron stores also increase the risk of type 2 diabetes (30). Women from the Nurses Study with high levels of ferritin (>107 ng/mL) were nearly 3 times more likely to develop type 2 diabetes over a 10-y interval, independent of other risk factors such as body mass index (BMI), age, and ethnicity. High levels of ferritin were a risk factor for the development of gestational diabetes mellitus (GDM) in pregnant women. Nonanemic gravidas from Hong Kong who developed GDM during the course of pregnancy were compared with controls without anemia or diabetes selected at random from the at-risk population. Unadjusted concentrations of serum ferritin, iron, transferrin saturation, and the post-natal hemoglobin were significantly higher at 28–31 wk gestation in cases with GDM compared with controls (31).

In Camden, use of iron supplements increased serum ferritin concentrations. At entry to care and in the 3rd trimester, gravidas who took iron were significantly more likely to be in the highest quintile of serum ferritin. At entry the likelihood of being in the highest quintile was increased by 44% (OR = 1.44, 95% CI 1.04–1.99) and in the 3rd trimester it was increased 2-fold (OR = 2.01, 95% CI 1.48–2.74). We were able to detect an association between maternal serum ferritin and gestational diabetes using data from >1023 gravidas from Camden. Controlling for potential confounding variables (age, BMI, parity, ethnicity, smoking, iron supplement use), we found a 2-fold increase in risk of GDM for women in the highest quartile of serum ferritin at entry (AOR 2.32; 95% CI 1.06–5.08) and nearly a 3-fold increase in the 3rd trimester (AOR 2.9; 95% CI 1.27–6.95) (32). This positive relation suggests that iron stores may play a role in the development of GDM, a precursor of type 2 diabetes mellitus.

Supplementation with iron clearly augments iron status and iron stores. Whether supplementation with iron during pregnancy increases oxidative stress by adding to iron stores and creating a temporary iron surplus has been little studied. Because an increase in oxidative stress is part of normal pregnancy, routine iron supplementation in women who were not iron depleted or deficient might also contribute to or exacerbate oxidative stress. Lachili et al examined the influence of an iron supplement and vitamin C, an antioxidant that increases iron absorption, on oxidative stress during pregnancy (33). They found that administration of an iron supplement (100 mg/d as fumarate) with vitamin C (500 mg/d as ascorbate) during the 3rd trimester of pregnancy increased measures of maternal iron status. An indicator of oxidative stress from lipid peroxidation, plasma TBARS, was significantly increased in the n = 27 supplemented women compared with controls (33).

We were able to confirm the presence of increased oxidative stress in association with increased iron stores during pregnancy (Scholl, Chen, and Stein, 2004, unpublished observations). In Camden there is ongoing research on oxidative stress. ~350 gravidas from the Camden cohort had urinary excretion of 8 hydroxydeoxyguanosine (8-OH-dG) measured with the Genox kit (Genox Corporation, Baltimore). In this sample, a high level of serum ferritin at entry (>59 ng/mL) was associated with a 2.7-fold (95% CI 1.40–5.41) increased risk of having 8-OH-dG in the highest tertile; in the 3rd trimester a similar relation was found between high transferrin saturation (>21.7%) and 8 OH-dG in the highest tertile (AOR = 3.3; 95% CI 1.28–8.11). Thus, preliminary findings suggest an association between increased iron stores and the excretion of 8-OH-dG, a marker of oxidative damage to DNA in the maternal-fetal unit.

Risks and benefits of increased maternal iron status and stores from prophylactic iron supplementation should be examined further. For example, it would be important to know if higher levels of ferritin among gestational diabetics and women who deliver preterm represent increased iron stores as opposed to inflammation, infection, or failure of the plasma volume to expand. If increased iron stores are implicated, then it may be appropriate to identify an upper limit for iron-replete pregnant women beyond which prophylactic supplementation is not indicated. While iron supplementation may improve pregnancy outcome when the mother is iron depleted, iron deficient or has IDA it is possible that prophylactic supplementation may increase risk when the mother is not. Anemia and IDA are not synonymous, even among low-income minority women in their reproductive years.

The author has no personal or financial conflict of interest related to this project.

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Menopause, micronutrients, and hormone therapy

Judith Wylie-Rosett

ABSTRACT
Micronutrient and herbal/phytochemical supplements are of increasing interest as potential alternatives to using estrogen therapy in treating menopausal symptoms. This article provides an overview of the questionnaires that assess menopausal symptoms and research efforts to better standardize symptom assessment. The reported rate of symptoms varies by ethnicity, stage of menopause, hormonal therapy and the measurement method. The use of estrogen therapy has declined sharply after the Women’s Health Initiative (WHI) Hormone Trial was stopped early because the potential risks outweighed potential benefits. There is a limited research base that addresses the efficacy of supplements in controlling menopausal symptoms. The generalizability of several studies is limited because the study participants experience menopause as the result of treatment for breast cancer. The article concludes with a review of guidelines and of issues that need to be addressed in future research studies with emphasis on questions related to clinical practice. Am J Clin Nutr 2005;81(suppl):1223S–31S.

KEY WORDS Menopausal symptoms, micronutrients, supplements, phytoestrogen, herbal supplements, estrogen, progestin, cancer, heart disease

INTRODUCTION
Micronutrient and herbal/phytochemical supplements are of increasing interest as potential alternatives to using estrogen therapy in treating menopausal symptoms. The objectives of this article are to address following 4 questions. (1) What is known about the symptoms of menopause? This will be addressed with an overview of various instruments that are used to assess menopausal symptoms and factors that are related to the prevalence of reported symptoms. (2) What is the current evidence regarding hormone (replacement) therapy? This will focus on the of the WHI Hormone Trial findings (1). How publication of the WHI findings are related to the decline in estrogen prescriptions will also be addressed. (3) What is known about micronutrients and phytochemical treatment in menopause? Studies of supplements will be reviewed, focusing on factors that may affect generalization of the findings. (4) What clinical and research questions need to be addressed now? The article will conclude with a review of factors affecting the design of studies, questions that need to be addressed, and guidelines for addressing menopausal symptoms. Clinicians can use such guidelines to develop an open dialogue and individual tailoring of advice for women during menopause based on risk profile and symptoms (2). The review of guidelines will also address limitations in the evidence base for recommending micronutrients and phytoestrogens/herbal supplements as possible alternatives to hormonal therapy for women who suffer from menopausal symptoms.

WHAT IS KNOWN ABOUT THE SYMPTOMS OF MENOPAUSE?

The term “menopause” comes from the Greek words meno (month) and pause (to end). Thus, the literal definition is the end of the cycle of monthly menstrual bleeding. Menopause develops as the result of decreased estrogen and a disruption of the hormonal cycle associated with ovulation. The absence of menstrual periods for 12 mo is usually used as the definition of “natural” menopause. The age at which menopause occurs varies widely ranging from late thirties to late fifties with the range for most women being between ages 48 and 55 y. Self-reported data from middle age women, who participated in the Behavioral Risk Factor Surveillance System (BRFSS) in Florida, Minnesota, and Tennessee, indicate that the most common symptom is hot flushes or flashes, which was reported by 60% of the women (3). Night sweats were reported by 48% of the women, and 41% reported trouble sleeping.

The perimenopausal transition lasts for about 4 y for most women experiencing natural menopause. Vasomotor menopausal symptoms commonly develop one to 2 y before the cessation of menstrual flow. A longitudinal study of menopausal women found that 46% of women report hot flushes and bouts of sweating in the year immediately preceding menopause. The proportion reporting vasomotor symptoms increased to 67% during the first year after menopause, and fell to 49% in the second postmenopausal year (4). An overview of how hormonal transition in menopause is related to occurrence of vasomotor symptoms is provided in Table 1. Hysterectomized women experience surgical menopause, which is often accompanied by an abrupt onset of vasomotor symptoms. Chemotherapy and radiation can also induce a fairly rapid onset of the vasomotor and other menopausal symptoms with ≈30% of women under 35 y of age experience ovarian failure premenopausal women after undergoing chemotherapy; the proportion increases to half for...
women age 35 to 40 y and to 75% to 90% for women over 40 y of age (5). In addition to age, the chemotherapy dosage is a major predictor of premature menopause. Treatment of menopausal symptoms in this group is particularly complex because of ongoing treatment and risk related to having cancer as well as the abrupt onset of symptoms.

A population based study conducted in Sweden, the Women’s Health in Lund Area study, found that after controlling for other variables, the frequency and severity of hot flushes was more almost 3-fold higher in women who had oophorectomy, was over 50% higher in women drinking large amounts of alcohol, and was 30% higher in women who gained weight. This study also assessed vaginal dryness, which is the symptom commonly associated with atrophy of estrogen-sensitive tissue, although urinary difficulties such as stress incontinence can also occur (6). For the postmenopausal women in this Swedish cohort, vaginal dryness was almost 2 fold higher in the women age 58-64 y than in women 50–53 y of age. However, having menopause later (after age 53 y) was associated with a lower likelihood of vaginal dryness than having menopause at earlier age (6). It appears that women who have menopause early may suffer more symptoms over a longer period of time, which may be linked to lower endogenous estrogen production or an earlier shutoff of estrogen production. Obesity and larger waist circumferences were associated with less vaginal dryness presumably due to higher estrogen and androgen hormones (6).

There are several standardized menopausal quality of life questionnaires that address psychosocial domains, vasomotor symptoms, and/or some urogynecological dimensions associated with menopause. Schneider (7) identified 5 standardized menopausal questionnaires that met the following 4 criteria: (1) factor analysis, (2) subscales measuring different aspects of symptoms, (3) sound psychometric properties, and (4) standardized among populations of women. The characteristics of these questionnaires are listed in Table 2. All of the questionnaires include some aspect of emotional well-being. Three of the questionnaires (the Greene Climacteric Scale, the Women’s Health Questionnaire, and the Menopausal Symptom List) include vasomotor symptoms, and only the Menopausal Rating Scale includes urogenital subscale. A recent paper suggested adding items to menopausal symptoms scales to assess voice impairment focusing on discomfort when talking and changes in voice quality because of the frequency of encountering these as clinical problems (8).

The Study of Women’s Health Across the Nation (SWAN) assessed menopausal symptoms in a multietnicic cohort of over 16 000 American women aged 40–55 y. The 5 ethnic/racial groups represented included African Americans, Chinese Americans, Hispanic American, Japanese Americans, and Euro American (described as white). The African American women had the highest frequency of hot flashes followed by Hispanic American and Euro American women, respectively. In the SWAN cohort, the Chinese and Japanese American women reported lower rates of hot flashes (9). The Hispanic American women were more likely to report heart pounding as a symptom. Ironically, quality of life ratings were not associated with frequency of symptoms. The Chinese and Japanese American women were less likely to view going into menopause as a positive experience and to expect to become irritable or depressed (10). Being less acculturated and speaking Cantonese or Japanese was associated with poorer scores for attitudes about menopause. However, the scores did not differ on the basis of acculturation or language preference among the Hispanic American women. Variables associated with increased reporting of hot flashes are summarized in Table 3.

### Table 2

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Subscales number and subscale names</th>
<th>Total item number</th>
<th>Rating measure points used in scoring</th>
<th>Reliability of subscales</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green Climacteric Scale</td>
<td>4 - Vasomotor, Somatic, Anxiety, Depression</td>
<td>21</td>
<td>Severity - 4 point</td>
<td>0.83–0.87</td>
</tr>
<tr>
<td>Women’s Health Questionnaire</td>
<td>8 - Vasomotor, Somatic, Anxiety, Depression, Cognitive, Sleep, Sex, Menstrual</td>
<td>32</td>
<td>Presence/absence - 2 points</td>
<td>0.79–0.96</td>
</tr>
<tr>
<td>Menopausal Symptom List</td>
<td>3 - Vasomotor, General somatic, Psychological</td>
<td>25</td>
<td>Frequency Severity - 3 points</td>
<td>0.74–0.82</td>
</tr>
<tr>
<td>Menopause Rating Scale</td>
<td>3 - Somatov egetative, Urogenital, Psychological</td>
<td>11</td>
<td>Severity - 3 points</td>
<td>0.6 (average?)</td>
</tr>
<tr>
<td>Utian Quality of Life Score</td>
<td>3 - Emotional, Occupational, Health, Sexual</td>
<td>23</td>
<td>Severity - 5 points</td>
<td>Not Available</td>
</tr>
</tbody>
</table>

Data for table derived from reference number 7.
WHAT IS THE CURRENT EVIDENCE REGARDING HORMONE (REPLACEMENT) THERAPY?

The emerging data from the WHI has dramatically reduced prescriptions for estrogen for postmenopausal women (11). Ironically, estrogen prescriptions for postmenopause was referred to as estrogen, or hormone, replacement therapy (HRT) until the findings of the WHI hormone trial indicated the risks outweighed the benefits. Indeed, terminology changed from HRT to hormone therapy (HT) after the early stopping of the WHI estrogen plus progestin trial in 2002 and the conjugated equine estrogen in 2004 (12, 13). The dramatic shifts in use of hormone therapy for postmenopausal women are quite remarkable.

A 1995 national population-based telephone survey of ~500 postmenopausal (50 to 74 y of age) women indicated that 37.6% of the women reported current estrogen therapy. The proportion was 3-fold higher for women who had undergone a hysterectomy compared with women who had not (~60 versus 20%) (14). Women in the South and the West were >2 and half times more likely to use HRT than women in the Northeast (14). Use was 4-fold higher in college graduates than those with lower education, and the frequency of use in women with diabetes mellitus was less than one-fifth that of women without diabetes (14). However, the frequency of hormone usage was not related to other cardiac risk factors and most psychological characteristics.

An examination of hormone trends in the United States from 1995 to 2003 by Hersh, Stefanick, and Stafford was derived from National Prescription Audit database and the National Disease and Therapeutic Index data (11). The total annual hormone prescriptions in the United States increased from 58 million in 1995 to 90 million with an annual increase of 15 million per year. The prescriptions remained stable until 2002 when the results of the WHI were released. The number of prescriptions dropped by 66% for Prempro (the estrogen plus progestin combination used in the WHI) and by 33% for Premarin (the conjugated equine estrogen [CEE] used in the WHI) (11). The total number of women for whom postmenopausal estrogen was prescribed rose from 10 million in 1995 to a peak of 15 million in 2001, and fell back to 10 million in 2003.

The WHI findings were foreshadowed by the Heart and Estrogen/Progestin Replacement Study (HERS), which examined the effect of hormone replacement therapy on the rate of recurrent heart problems in women who already had heart disease and was published in 1998 (15). Generalizing from the HERS results was difficult due to the small sample size and eligibility criteria. The WHI included over 27,000 women, which was 10 times the number of women included in the HERS. In addition, HERS inclusion criteria included having had previous myocardial infarction and exclusion criteria included having had a hysterectomy. As the results of these stringent eligibility criteria, only 2% of the WHI Hormone Trial participants would have qualified for HERS.

The overall findings of the WHI are summarized in Table 4 (12,13,16–20). In the Estrogen-plus-Progestin study, women taking combined estrogen plus progestin had more heart attacks, strokes, blood clots, and breast cancers compared with women taking placebo. Women taking estrogen plus progestin also had fewer colorectal cancers and hip fractures, and there was no effect on the number of deaths. The increased risk of stroke and the decreased risk of fractures were also seen in women taking CEE (18) alone, which could raise questions about whether the effects were due to estrogen per se. However, the findings that CEE did not increase breast cancers or decrease colorectal cancers were different from the estrogen-plus-progestin results. Continued analyses are trying to determine the reasons for the differences. Women who have a hysterectomy may differ in other health characteristics compared with women who have not. Alternately, progestin may change disease risks and benefits when added to CEE.

On the benefit side, women taking CEE had fewer hip fractures. For other diseases of interest, the picture was not clear. Either there were no effects of CEE, or the data were not strong enough for scientists to be sure they were real. For example, the unexpected finding that fewer women taking CEE had breast cancer needs further study. The effect of CEE on blood clots was also uncertain. The CEE appeared to have a neutral effect on heart attacks, colorectal cancer, and death because the differences were very small.

The WHI results indicate that for every 10,000 women taking CEE, 12 more women per year would have strokes; the overall stroke incidence in the WHI was 44 women in the CEE group compared with 32 women in the placebo group. For deep vein thrombosis (DVT), the increase was 6 more per 10,000 per year. It is not clear if more women taking CEE had increased risk of pulmonary emboli. The mixed results of the WHI with respect to risk and benefits on hormones has resulted in the need for a greater dialogue between clinicians and patients to evaluate the potential risks and benefits on an individual basis.

WHAT IS KNOWN ABOUT MICRONUTRIENTS AND PHYTOCHEMICALS IN MENOPAUSE?

Hot flashes, which are the primary symptom of menopause, appear to be caused by dysfunction of the central thermoregulatory system when estrogen availability is decreased. Norepinephrine and serotonin pathways are thought lower the low the set point for the thermoregulatory nucleus, which allows heat loss to be regulated by a subtle change in core temperature. Hot flashes are preceded by a rise in norepinephrine, and injecting norepinephrine can induce an elevation in the core temperature. The elevation in core temperature by these hormones is thought to be counterregulated by endorphin and catecholesterol, which are metabolic byproducts of estrogen and other sex hormones. These complex interrelationships are illustrated in Figure 1 (21). Micronutrients or phytochemicals should be examined with respect to how they may have impact on this proposed model of thermoregulation. Phytoestrogens need to be examined for their estrogenic function and their potential to bind receptors and thereby block the normal pathways by which estrogen regulates thermoregulation. Herbal supplements that may have serotonin receptor uptake inhibitory functions are also being examined for potential use in thermoregulation.

There are over 40 manufacturers that market herbal products for menopause symptom control (22), but to date little is known about the efficacy. Behavioral Risk Factor Surveillance System (BRFSS) data indicate that 46% of women have used complementary or alternative therapy for menopausal symptoms (3). The alternative approaches, which included herbal and, to a lesser extent, micronutrient supplements, were often used in combination with conventional approaches prescribed or recommended by their health care provider (23). Women with more severe symptom ratings were more likely to use alternative/
complementary approaches. About one-third of women reported not using either alternative/complementary or conventional approaches for menopausal symptoms. Another survey found that only a little over half of women who used herbal products for menopausal symptoms indicated that their physician was aware of their usage of herbal products (3, 23).

Baseline data from participants in the SWAN study indicate that 48.5% had used at least one complementary or alternative therapy during the preceding year (24). Complementary and alternative therapy users were more likely to be younger, have more education, be white, and reside in California than the non-users. Herbal therapies were more likely to be selected by the Chinese American women than other groups, but emotional stress and anxiety were associated with herbal therapy among the Japanese American women as well (24).

A 1999 National Institutes of Health workshop identified knowledge that should be researched to provide an adequate evidence base to assess the growing public interest in phytoestrogens and other products for menopausal symptoms and other health issues for older adults (25). Several recent reviews, editorials, and commentaries have addressed the evidence base for alternative and complementary medicine focusing on the interest generated by increased caution in using estrogen therapy (26–33). Micronutrient and herbal supplements that are commonly used to treat menopausal symptoms include vitamin E, black cohosh, soy, and other phytoestrogens, which are used to treat the vasomotor symptoms. Other herbal treatments such as ginko biloba, ginseng, and St. Johns wort have been used for mood related symptoms, and valerin has been used for sleep disturbances associated with menopause, but these issues are beyond the scope of the present review. Interpretation of study findings is hampered by the small sample size, variability in the product tested (especially for the soy), and the clinical characteristics of the study population (menopause induced by treatment of breast cancer). With the decline in popularity of postmenopausal hormone therapy, the clinical and research interest to address the effects of alternative/complementary on menopausal symptoms is likely to increase dramatically. Research that focuses on micronutrient and related treatments is likely to increase to provide an evidence base to address questions posed by menopausal women and their care providers.

VITAMIN E

Although Vitamin E is widely used for treating hot flashes, the research database is extremely limited (26). A randomized crossover trial (4 wk per treatment condition) conducted in 120 women treated for breast cancer found that Vitamin E (800 IU) resulted in 1 fewer hot flash per day than the placebo (34). Despite the lower occurrence of hot flashes, an end of study survey indicated a low interest in using vitamin E for hot flashes (34). Whether the findings can be generalized to women undergoing naturally occurring or surgical menopause remains to be determined. Controlled studies are needed to assess the effects of vitamin E on symptoms using one of the standardized menopausal symptom questionnaires and on hormones affected by

FIGURE 1. Heavy lines denote key pathways involved in promoting or inhibiting the adjustment of core temperature via hot flash reaction triggered by the hypothalamus. Dashed lines indicate reactions that inhibit hot flash reactions. Adapted from reference 4.
menopause. Research also needs to address how vitamin E affects the metabolic pathways involved in the hot flash reaction.

**BLACK COHOSH (CIMICIFUGA RACEMOSA, BLACK SNAKERoot)**

Black cohosh, botanically a member of the buttercup family, has been widely used in Native American therapy for a variety of ailments including dysmenorrhea and labor pains as well as for the treatment of menopausal symptoms (35–40). Concentrated extracts of black cohosh is sold as a standardized product. Remifemin® is the product most widely used in reach studies, although a number of companies produce black cohosh extract. The formulation of Remifemin has changed over time, but currently, 1 tablet contains black cohosh extract corresponding to 20 mg of crude drug standardized to 1% 27-deoxyacetin (35). It has been difficult to discern the effects of black cohosh despite the availability of a standardized formulation and reported findings from 3 of 4 randomized trials indicating a reduction in menopausal symptoms (27, 35). The black cohosh treatment regimen was confounded by other treatments or open label treatment (27, 35).

Black cohosh contains a number of compounds with potential bioactivity including triterpene, glycosides, resin, salicylates, isofurutaric acid, sterols, and alkaloids. Analysis of black cohosh from various woodlands in the Eastern United States and Remifemin yielded no formononetin, the phytoestrogen thought to account for the reported reduction in menopausal symptoms (27, 39). While black cohosh contained a standard quantity of 27-deoxoactin, little is known about the variability in other potentially bioactive compounds that have been found in analyses of black cohosh samples. Many of these compounds may have potential benefits, but potential risks also need to be considered. Women with breast cancer are often advised to avoid black cohosh due to the lack of evidence to conclude that it is safe (36).

The mechanism of action by which black cohosh reduces hot flashes is not understood. Black cohosh does not appear to alter the hormonal pattern associated with menopause, low estrogen accompanied by elevated luteinizing hormone (LH), and follicle-stimulating hormone (FSH). The effects of black cohosh on vaginal tissue are also not clear (37). It is possible that it affects the pathway down stream from the estrogen. The National Center of Complementary and Alternative Medicine at the NIH has funded a randomized, controlled clinical trial to determine whether black cohosh reduces the frequency and severity of hot flashes and other menopausal symptoms (37). Additional rigorously controlled studies are needed to ascertain the true effects of black cohosh on menopausal symptoms and to make an evidence-based decision regarding who may benefit and for whom its use the risks are likely to outweigh any potential benefits (35).

**PHYTOESTROGENS FROM SOY, RED CLOVER, AND FLAX**

The phytoestrogens that have been isolated from a variety of plant food are phenolic (rather than steroidial) compounds; the major categories of phytoestrogens include isoflavones, lignans, and coumestans (33). Phytoestrogens can function as selective estrogen receptor modulators (SERMs) by binding to receptors for estrogen metabolites. SERMs may function as estrogen metabolites in some tissues but not in others. While laboratory-developed SERMs are widely used in treating women with estrogen receptor positive cancers, less is known about the SERM function of phytoestrogens. Soy, other beans, clover, and alfalfa contain isoflavone precursors, which are converted to genistein, daidzein, and equol by intestinal bacteria. Flax seeds, other seeds, legumes, whole grains, and some fruits and vegetables contain lignan precursors that can be converted to enterolactone and enterodiol by intestinal bacteria. The phytoestrogens can have estrogenic activity as potential dietary derived modulators with endocrine function.

To date, studies have been small and lack statistical power, and these studies cannot be readily aggregated to increase the statistical power due to differences in their methodologies. In the systematic review by Kronenberg and Fugh-Berman, only 3 of the 12 randomized controlled trials found that soy phytoestrogen supplements or soy products reduced the frequency or severity of hot flashes. In one of the studies the decrease in hot flashes was accompanied by increases in 17β-estradiol and decreases in total and LDL cholesterol (27). Flax seeds have been reported to have estrogenic, antiestrogenic, and steroid-like activity (22).

Although the phytoestrogen in soy is often used by women who are trying to control menopausal symptoms, a recent study found that 25 g of flax seed alters metabolism of estradiol in favor of 2-hydroxyestrone, which is less biologically active (41). Theoretically, the metabolic shift could be accompanied by a reduction in menopausal symptoms. However, there are no published studies that have focused on the potential of flax seed in controlling menopausal symptoms. A soy beverage did not reduce the hot flash score (frequency and severity) to any greater extent than the placebo in a study involving women with breast cancer (42).

Red clover contains the phytoestrogen formononetin, biochanin A, daidzein, and genistein. Isoflavone and other supplements derived from red clover are readily available. The overall finding of research to date is that red clover and its related supplements are not better than placebo in controlling hot flashes. Promensil (total isoflavone content 40 mg per tablet), which was not found to be beneficial in controlling menopausal symptoms in a study that tested the 40-mg dosage, was found to reduce moderate to severe symptoms at 80 mg per day (43). Promensil was also not found to affect breast tissue density, unlike estrogen, which increases density, and tamoxifen, which reduces density. How the phytoestrogens interact with SERMs needs to be evaluated (44). More research is also needed to address how commonly eaten foods that contain phytoestrogens (fennel, celery, parsley, nuts, whole grains, apples, and alfalfa) affect menopausal symptoms as well as addressing the effects of supplements.

**OTHER HERBAL SUPPLEMENTS**

Dong quai is prepared as a tonic in traditional Chinese medicine, and is unclear if it contains compounds that have estrogenic properties (45). There are little data on the effects of dong quai because in traditional Chinese medicine it is usually in combination with other herbs. The only published randomized controlled trial found that dong quai was no better than the placebo in reducing menopausal symptoms (45).

There are no controlled studies on the other herbal supplements that are sometimes recommended for menopausal symptoms. Evening primrose oil, which is a rich source of gamma linolenic acid, an intermediate compound between cis-linolic acid and prostaglandin, is used to treat mood swing, irritability, and breast tenderness associated with premenstrual syndrome and menopause. Wild yam is also used to treat a variety of
TABLE 3  
Assessment of variables associated with increased reporting of hot flashes

<table>
<thead>
<tr>
<th>Factors related to the association</th>
<th>Medical History</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal History</td>
<td>Genetic factors may play a role in the vasomotor symptoms of menopause.</td>
</tr>
<tr>
<td>Early age of menopause onset and early age of menarche</td>
<td>Possible factors may include genetic and/or hormonal factors that may cause early onset of menarche and menopause as well as the severity and frequency of menopausal symptoms.</td>
</tr>
<tr>
<td>History of Irregular Menses</td>
<td>Hormonal factors may account for the link between menopausal symptoms and irregular menses.</td>
</tr>
<tr>
<td>Higher BMI</td>
<td>Body fat may impair ability to dissipate heat, but estrogen may be higher as well.</td>
</tr>
<tr>
<td>Dietary and environmental triggers</td>
<td>Vasodilation accompanied by elevation of the core temperature.</td>
</tr>
<tr>
<td>Alcohol</td>
<td>Circulatory impairment</td>
</tr>
<tr>
<td>Cigarette Smoking</td>
<td>Impairs ability to dissipate heat</td>
</tr>
<tr>
<td>Hot/Humid Weather</td>
<td>Possible changes in hypothalamic signals resulting in vasodilation accompanied by elevation of core temperature.</td>
</tr>
<tr>
<td>Sulfites, Mono sodium glutamate (MSG), Sodium Nitrate</td>
<td>Effects not consistently reported.</td>
</tr>
</tbody>
</table>

1 Based on data presented in references 6 and 9.


drinual problems and menopausal symptoms. American ginseng is sometimes used for menopausal symptoms because it is considered to have a cooling effect, while Asian ginseng is considered a heat-raising tonic (46). Other herbs that are used for their potential effects on mood and may alter the serotonin pathway, which are sometimes recommended to menopausal women, include kava kava, St Johns wort, and sage. Flavonoids are also of interest because they appear to have a weak estrogenic effect, but the data are limited to 1 study potentially promising study testing herperidin from citrus fruit in combination with vitamin C (26). An overview of the potential benefits and risks of micronutrients and phytochemicals used for menopausal symptoms is presented in Table 5.

WHAT CLINICAL AND RESEARCH QUESTIONS NEED TO BE ADDRESSED NOW?

Clinicians face a considerable challenge in providing individually tailored assessments of the potential risks and benefits of estrogen therapy and the complementary and alternative options to estrogen. The concept of “shared decision making balance for evidenced-based patient choice” has emerged as health care providers are rethinking how to discuss estrogen therapy with women during and after menopause (47). Alternative options that women in one survey mentioned include: herbal preparations/vitamin supplements, healthy living (diet/exercise) and mind/body practice (prayer and mental healing). The range of options mentioned did not vary by race, ethnicity, or surgical status.

The 2003 position statement from North American Menopause Society addressed alternatives to estrogen therapy. The specific recommendations included, “First consider lifestyle changes, either alone or combined with a nonprescription remedy, such as dietary isoflavones, black cohosh, or vitamin E. For moderate to severe menopause-related hot flashes estrogen-containing treatments or progestogens, venlafaxine, paroxetine, 

<table>
<thead>
<tr>
<th>Outcome condition</th>
<th>Estrogen alone</th>
<th>Estrogen plus progestin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocardial Infarction</td>
<td>No significant difference found between treatment arms.</td>
<td>Risk was 29% higher than in the placebo group</td>
</tr>
<tr>
<td>Stroke</td>
<td>Risk was significantly higher than in placebo group with an increase of 12 strokes per 10,000 person years</td>
<td>Risk was 41% higher than in the placebo group</td>
</tr>
<tr>
<td>Blood clots</td>
<td>Did not appear to increase or decrease risk.</td>
<td>The overall rate of increase was two-fold higher compared to the placebo group. Compared with those on placebo, risk was fourfold during first 2 years.</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Trend for possible reduction</td>
<td>Risk increased by 24 percent after 4 years, regardless of age, family history or race</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>No difference found between treatment arms.</td>
<td>Risk was 37% lower than in the placebo group.</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>Not applicable</td>
<td>Trend for higher rate than in the placebo group, which was not statistically significant.</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>There were 6 fewer hip fractures per 10,000 person years than in the placebo group.</td>
<td>Hip fractures were 34% lower and total fractures were 24% lower than in the placebo group.</td>
</tr>
<tr>
<td>Cognitive impairment</td>
<td>Trend for higher rates of dementia than in the placebo group, which was statistically significant.</td>
<td>Women on all forms of hormone replacement therapy had increased risk of mild cognitive impairment or probable dementia.</td>
</tr>
<tr>
<td>Quality of life</td>
<td>No differences found between treatment arms.</td>
<td>Some trends noted but no clinically significant effects on physical or emotional health, pain, energy, sleeping or sexual satisfaction</td>
</tr>
</tbody>
</table>

1 Data derived from the Women’s Health Initiative (reference numbers 1, 12, 13, 16–20). Adapted from R Rabin, Newsday, June 23, 2004.
Menopause, Micronutrients, and Hormone Therapy

TABLE 5
Overview of potential benefits and risk: use of micronutrients and phytochemicals for menopausal symptoms

<table>
<thead>
<tr>
<th>Supplement dosage evaluated</th>
<th>Potential active compound(s)</th>
<th>Evidence of potential benefits</th>
<th>Potential risk or side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E 800–1200 IU²</td>
<td>α tocopherol γ tocopherol</td>
<td>One trial reporting reduction of hot flashes by one per day in 4-week placebo controlled cross-over study</td>
<td>Can enhance the effects of anticoagulation medications.</td>
</tr>
<tr>
<td>Black Cohosh 40 mg/1 or 2 times per day or liquid¹ (standardized product available from 16 companies.)</td>
<td>Formononetin standardized on the basis of 27-deoxactin activity.</td>
<td>Four randomized trials with three of the four reporting reduction is symptom severity or frequency. All studies had potential confounding by other medications.</td>
<td>Reported side effects include rash, gastrointestinal disturbances, and individual case reports of liver damage or failure. Risks for short-term therapy appear to be low.</td>
</tr>
<tr>
<td>Dong Quai 4.5 g in three divided does daily</td>
<td>Does not appear to have phytoestrogen activity.</td>
<td>One randomized trial reported no effect on symptoms. Dong Quai has customarily been used in combination with other Chinese herbs.</td>
<td>Dong quai contains coumarins that can enhance the effects of anticoagulation medications and furocoumarines that result in photosensitivity.</td>
</tr>
<tr>
<td>Soy</td>
<td>Isoflavones (genistein, diadzein)</td>
<td>Three of 12 randomized trials reported reduction in frequency or severity of hot flashes.</td>
<td>Concentrated supplements containing phytoestrogens could pose risks associated with estrogen therapy.</td>
</tr>
<tr>
<td>(Evening Primrose OIl) 2 gm twice daily</td>
<td>γ linolenic acid (often commercially formulated with Vitamin E)</td>
<td>No benefit was demonstrated in a clinical trial testing it in combination with 40 mg vitamin E.</td>
<td>Side effects and risks appear to be low.</td>
</tr>
<tr>
<td>Red Clover Promensil (82 mg isoflavone) and Prmosteil (57 mg isoflavone)</td>
<td>Phytoestrogens (formononetin, biochanin A, daidzein, genistein)</td>
<td>Clinical trials have not found evidence of reduction in menopausal symptoms.</td>
<td>Side effects and risks appear to be low.</td>
</tr>
</tbody>
</table>

¹ One trial conducted in breast cancer patients (reference 34).
² Remifemin as liquid or tablets (GlaxoSmithKline, Philadelphia, PA) in four studies.

Fluoxetine, or gabapentin. Clinicians are advised to enlist women’s participation in decision making when weighing the benefits, harms, and scientific uncertainties of therapeutic options” (2).

On the other hand, clinicians are urged to recommend medical alternatives to estrogen and to be more directive and less collaborative in advising patients. The 2003 review by Amato and Marcus focused on the inadequacy of the evidence base with regard to dietary supplements, stating “Phytoestrogens and black cohosh appear to be safe when used for short periods of time, much larger and longer studies are needed to detect infrequent but potentially serious adverse events...Women who do not wish to take hormone therapy to treat menopausal symptoms should be encouraged to consider using selective serotonin reuptake inhibitors and other conventional therapeutic options” (48).

Clearly a wide range of options can be used to treat menopausal symptoms. The dialogue should begin with eliciting the potential symptoms. For women who are encountering depression, which may or may not be directly related to menopause, the treatment needs are quite different from a woman whose mood is upbeat but she is experiencing hot flashes. An open dialogue can help achieve a solution that is acceptable to each woman who has symptoms. Questions of research interest that also need to be addressed in the clinical dialogue include:

1. How do risks for heart disease, breast cancer, and osteoporosis influence decision-making with regard to taking estrogen for each postmenopausal woman?
2. Which postmenopausal women currently on estrogen therapy should stop?
3. What should be anticipated as side effects associated with stopping estrogen therapy?
4. What long-term safety issues need to be considered if a women on each of the alternative therapies?

CONCLUSIONS

Menopausal symptoms are common, but vary by BMI, ethnicity, smoking status, and other lifestyle variables. The results of the WHI have altered the assumption that estrogen therapy could reduce cardiovascular risks that rise after menopause. With the decline in prescriptions for estrogen, the potential role of micronutrients and phytochemicals in controlling menopausal symptoms is of increased interest. Vitamin and phytoestrogens (eg, black cohosh, soy) may be beneficial, but studies have variable results. Collaborative decision making can help women with hot flashes reduce symptoms.

The declining usage of estrogen therapy is likely to increase research that addresses how micronutrients, phytoestrogens, and other food/herb derived compounds affect menopausal symptoms. The scientific rigor of studies needs to be improved so that the benefits and potential risks of the treatment options for menopausal symptoms are better delineated. Research questions that need to be addressed include:
What are the effects of a combined micronutrient and phytoestrogen dietary approach? How does the metabolism of micronutrients and phytochemicals relate to their potential benefit in preventing or treating hot flashes and other menopausal symptoms? It is reasonable to test the potential maximal effect that can be achieved by combining the wide variety of foods containing phytoestrogens with supplements such as black cohosh and vitamin E. The combined effect of these foods needs to be evaluated. The research design for such a study could be patterned after the study by Jenkins et al, which tested a portfolio diet that combined cholesterol lowering dietary approaches (49). Combining dietary approaches with other lifestyle approaches such as physical activity, and light weight clothing is also worthy of study. What are the potential safety issues/adverse effects of phytoestrogen supplements? What are the long-term effects? How do they affect cancer risk in women with a personal or family history of estrogen positive breast cancer? Human and animal model research needs to evaluate potential safety issues for women with estrogen receptor positive cancers.

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Osteoporosis: the role of micronutrients

Jeri W Nieves

ABSTRACT
Osteoporosis and low bone mass are currently estimated to be a major public health threat. Adequate nutrition plays a major role in the prevention and treatment of osteoporosis; the micronutrients of greatest importance are calcium and vitamin D. Calcium has been shown to have beneficial effects on bone mass at all ages, although the results are not always consistent. Higher doses than the current US recommendation (600 IU) of vitamin D in the elderly (age ≥ 65 y) may actually be required for optimal bone health (800–1000 IU/d). The elderly can clearly benefit from increased vitamin D intakes; however, the potential importance of vitamin D in peak bone mass is just being investigated. Vitamin D has been related to falls, with supplementation reducing the number of falls. There are clear fracture benefits demonstrated in randomized clinical trials of calcium and vitamin D supplementation. The other micronutrient needs for optimizing bone health can be easily met by a healthy diet that is high in fruits and vegetables to ensure adequate intakes for magnesium, potassium, vitamin C, vitamin K, and other potentially important nutrients. Healthcare professionals need to be aware of the importance of adequate calcium and vitamin D intakes (easily monitored by serum 25(OH)D) for optimal bone health, as well as the prevention of falls and fractures. In addition, a healthy diet that includes 5 servings a day of fruits and vegetables should optimize the intake of micronutrients required for bone health. Am J Clin Nutr 2005;81(suppl):1232S–9S.

KEY WORDS Calcium, vitamin D, falls, fractures, osteoporosis, micronutrients

INTRODUCTION
The most recent definition of osteoporosis is a disease characterized by loss of bone mass, accompanied by microarchitectural deterioration of bone tissue, which leads to an unacceptable increase in the risk of skeletal failure (fracture). Osteoporosis and low bone mass are currently estimated to be a major public health threat for almost 44 million US men and women aged 50 and older, or 55% of the population in that age range (1). In fact, 1 in 2 women and 1 in 4 men over the age of 50 will fracture at some point in their lifetime. The costs to the healthcare system associated with osteoporotic fracture are ~$17 billion dollars annually (2), with each hip fracture having total medical costs of $40 000.

Adequate nutrition plays a major role in the prevention and treatment of osteoporosis; the nutrients of greatest importance are calcium and vitamin D. Numerous studies have shown that higher calcium intake at various ages is associated with higher bone mineral density compared with the bone mass of those with lower calcium intakes (3). In older postmenopausal women, the benefits of vitamin D and calcium supplementation in preventing bone loss, decreasing bone turnover, and decreasing nonvertebral fractures are clear (4).

An inadequate intake of either calcium, vitamin D, or both will influence calcium-regulating hormones. A deficiency of either calcium or vitamin D will result in reduced calcium absorption and a lower concentration of circulating ionized calcium. When this occurs, parathyroid hormone (PTH) secretion is stimulated and there is a resulting increase in PTH levels. The cumulative effect of higher PTH levels, secondary to poor calcium and vitamin D nutrition (secondary hyperparathyroidism), is an increase in bone remodeling leading to significant loss of bone and an increased fracture risk. Vitamin D supplementation, often in combination with calcium, appears to reduce the degree of secondary hyperparathyroidism associated with poor nutrition.

The recommended calcium intake changes with age and the current recommended intakes are listed in Table 1 (5). One of the highest daily intakes is required after age 50. Important dietary sources of calcium are dairy products (milk, yogurt, cheese), dark green vegetables; canned fish with bones (but not fish fillets); nuts; and more recently, fortified foods (including juices, waffles, cereals, crackers, and snack foods). The average US diet contains only 600 mg calcium a day and thus falls far below the recommended intakes (6). If an adequate calcium intake is not possible in the diet, a calcium supplement may be required and should optimally be taken in doses < 500 mg at a time to maximize absorption, because absorption decreases with greater calcium loads. The preferred time to take most supplements is with meals, because calcium is better absorbed with food. Calcium carbonate has more calcium per tablet (40%) than some of the other forms of calcium such as calcium citrate (23%). In most healthy individuals calcium intakes up to 2500 mg/d are safe (5).

In younger individuals, vitamin D synthesis in the skin is the primary determinant of serum 25(OH)D levels; however, the cutaneous synthesis is reduced in the elderly. Elevations in serum PTH and greater bone loss are often associated with lower levels of 25(OH)D. Vitamin D insufficiency is believed to play a strong

1 From the Clinical Research and Regional Bone Centers, Helen Hayes Hospital, West Haverstraw, NY, and Epidemiology, Mailman School of Public Health, Columbia University, New York, NY.

2 Supported by National Institute of Aging (AG1406).


4 Address reprint requests and correspondence to Jeri W Nieves, Clinical Research Center, Helen Hayes Hospital, Route 9W, West Haverstraw, NY 10993. E-mail: jerinieves@mindspring.com.
TABLE 1
Food and Nutrition Board Dietary Reference Intakes (Recommended Average Intakes for Calcium and Vitamin D)

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<th>Age (y)</th>
<th>Calcium (mg)</th>
<th>Vitamin D (IU)</th>
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<td>3–8</td>
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role in osteoporosis. The current US recommendation for vitamin D intake in people age 51 to 70 y is 10 μg/d (400 IU/d) and over age 70 y is 15 μg/d (600 IU/d; see Table 1 (5)). However, higher doses of vitamin D (800–1000 IU/d) in the elderly (age ≥ 65 y) may actually be required for optimal bone health, because these vitamin D doses have been shown to reduce fracture risk in this population (3, 4). Rich sources of vitamin D include fatty fish, fish-liver oils (cod liver oil), and liver. Several foods are also fortified with vitamin D including milk, margarine, orange juice, and cereals. There is general agreement that the serum levels of 25(OH)D are the best indication of adequate and inadequate vitamin D levels (7, 8).

In the United States, in the cohort 65 y and older in national health and nutrition examination survey (NHANES) III, 32% of whites, 64% of blacks and 53% of Hispanics had levels of 25(OH)D < 54 nmol/L, the median for the entire NHANES cohort (9). In a recent consensus conference, it was suggested that adequate 25(OH)D levels may be 80 nmol/L or 32 ng/mL, and that intakes of vitamin D of over 1000 IU per day are needed to achieve these serum levels (7). Vitamin D adequacy is often defined in older adults as the level of 25(OH)D needed to maximally suppress PTH levels. Serum 25(OH)D was the most significant (negative) determinant of serum PTH in a study of almost 1000 postmenopausal women (10). The rise in serum PTH appeared to start when serum 25(OH)D fell < 80 nmol/L. These data further suggest that the optimal level of serum 25(OH)D in postmenopausal women may be at least 80 nmol/L.

Suboptimal serum Vitamin D levels are widespread and should be evaluated, particularly in the elderly. However, the following populations are at particularly high risk of vitamin D deficiency: patients with malabsorption syndromes; patients with liver or kidney diseases; patients taking certain medications that interfere with vitamin D metabolism including steroids, dilantin, and phenobarbital.

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VITAMIN D AND BONE MASS

There have been several studies of vitamin D supplementation, typically in combination with calcium (500 to 1200 mg/d). The % difference between treatment with vitamin D and placebo resulted in average differences of 1.0%, 1.2%, and 0.2% respectively for the spine, femoral neck, and forearm (25–28). The prevalence of serum 25(OH)D deficiency and peak bone mass has been recently investigated. In this Finnish study, the median levels of serum 25(OH)D were 44, 24, and 41 nmol/L in July, winter, and again in July. These data indicate a fair number of young men aged 18–20 have low serum 25(OH)D. The authors found a positive correlation between serum 25(OH)D and bone mineral content at all sites (29). This study points to the potential need for intervention studies on the effects of vitamin D supplementation on the attainment of peak bone mass.
A French study reported that supplementation with 500 mg calcium and 400 IU of vitamin D given to women with serum 25(OH)D < 12 ng/mL, compared with placebo significantly decreased PTH and markers of bone turnover and improved bone mineral density (BMD). In this study, short-term changes in bone resorption markers can predict long term variations in bone density in elderly women with vitamin D insufficiency receiving vitamin D and calcium (30).

In another study of younger women (1 to 10 y postmenopausal), who had normal 25(OH)D levels (mean 82 nmol/L), the addition of 10,000 IU vitamin D per week to calcium supplementation at 1000 mg per day did not confer any benefits on BMD beyond that which was achieved with calcium supplementation alone (31). Although the elderly clearly require supplementation, in younger populations, there may be less benefit of vitamin D supplementation if serum 25(OH)D levels are normal.

**IMPACT OF CALCIUM AND VITAMIN D ON FALLS IN THE ELDERLY**

Falls are often the cause of hip fracture, which may result in death, morbidity, and admission to a nursing home. Clearly muscle strength, in particular lower extremity, should be one of the factors assessed and treated in older persons at risk for falls (32). In a recent analysis of NHANES data, in both active and inactive ambulatory elderly subjects, there was a strong improvement in lower extremity function based on walking speed and sit-to-stand speed, in serum 25(OH)D levels between 5 and 40 nmol/L, with continued but less significant improvement up to 90 nmol/L (9). Clearly vitamin D supplementation should be an important part of fall prevention, which in turn may reduce osteoporotic fractures. Adults with vitamin D deficiency have muscle weakness and are more likely to fall (33). In a meta-analysis (34), vitamin D supplementation appeared to reduce falls by 20%, and furthermore if 15 patients were treated with vitamin D, 1 fall could be prevented.

**PREVENTION OF FRACTURE WITH CALCIUM AND VITAMIN D**

A review of 16 observational studies assessing hip fracture and calcium intake found that an increase in usual calcium intake of 1 g a day was associated with a 24% reduction in the risk of hip fracture (35). A recent prospective cohort study did not show an association between dietary calcium and vitamin D intake and fracture in a cohort of Swedish women aged 50–85 y(36). In a follow-up of the cohort from the Nurses Health Study (37), an adequate vitamin D intake was associated with a lower risk of hip fracture, although neither milk intake nor a high calcium diet were associated with hip fracture reduction. A meta-analysis of observational studies relating calcium intake to fracture risk (38) also failed to show any association between calcium and hip fracture, although there was a suggestion that individuals with extremely low calcium intake may be at increased fracture risk.

However, data from randomized trials are much less prone to bias than the previously discussed observational studies. Two randomized clinical trials, which evaluated calcium supplementation found vertebral fractures to be reduced by 28% and symptomatic fractures to be reduced by 70% in the calcium supplemented group (39–40). Significant reductions in fracture (26 to 54% reduction in hip and non-spine fracture rates) have also been seen in those randomized clinical trials where calcium was given in conjunction with vitamin D (25–27, 41–42). In the studies where fractures were not reduced, the participants had higher baseline serum 25(OH)D levels, were not given additional calcium, or were given a lower dose of vitamin D (400 IU; 28; 43). One important study, published this year, was a randomized double blind controlled trial of 100 000 IU oral vitamin D3 (cholecalciferol) supplementation or matching placebo every 4 mo over 5 y in 2037 men and 649 women aged 65–85 y. The supplements were provided by mail every 4 mo. After 5 y, 268 men and women had incident fractures, of which 147 had fractures in common osteoporotic sites (hip, wrist, or forearm, or vertebrae). Relative risks in the vitamin D group compared with the placebo group were 0.78 (95% CI 0.61 to 0.99, P = 0.04) for any first fracture and 0.67 (0.48 to 0.93, P = 0.02) for first hip, wrist or forearm, or vertebral fracture. This study demonstrated that a widespread public health effort could be successful at preventing fractures without adverse effects in men and women living in the general community (44). Another important public health study of 9605 community dwelling Danish residents reported that 400 IU vitamin D and 1000 mg of calcium supplements significantly reduced fracture by 16% in this Northern European region that is known to be deficient in vitamin D (45).

A meta-analysis found that vitamin D decreased vertebral fractures and may decrease nonvertebral fractures (46).

To sustain the benefits of increased calcium and vitamin D the higher intake of these nutrients must be maintained. In a 2-year follow-up of participants in the Dawson-Hughes trial, the bone density gains were lost and bone turnover markers increased (47).

**PHOSPHORUS**

Phosphorus intake does not seem to influence skeletal homostasis within normal ranges of intake (RDA 700 mg/d), although excessive intakes particularly when combined with low calcium intake may be deleterious (48). Alternatively, adequate phosphorus intake is essential for bone building during growth and low serum phosphate will limit bone formation and mineralization (49). Foods that are high in phosphorus are milk, milk products, poultry, fish, meat, eggs, grains and legumes, and sodas, with only milk (and milk products) also having high amounts of calcium. High phosphorus intakes in the face of low calcium intake may lead to secondary hyperparathyroidism and bone loss. A diet adequate in calcium, with moderate protein and sufficient phosphorus was related to higher bone density (50). Phosphorus deficiency may be a marker of general nutritional inadequacy, similar to protein deficiency seen in the elderly, and in that regard could lead to an increased risk of fracture. These low phosphorus intakes or negative phosphorus balance due to food phosphorus being bound to supplemental calcium may create a relative phosphorus deficiency, which could limit osteoblast function and enhance osteoclastic bone resorption (51). At any age, the ratio of phosphorus to calcium is probably more important than the intake of phosphorus alone (51–52).

**SALT**

Sodium causes an increase in renal calcium excretion. The mean urinary calcium loss is 1 mmol per 100 mmol sodium (53). If absorbed calcium is less than the amount needed to offset these
obligatory calcium losses that are related to sodium intake, then bone mass will be negatively impacted. In observational studies, higher salt intake leads to higher levels of PTH and greater rates of bone resorption in postmenopausal women and men (54, 55). Furthermore, those with low calcium and high salt diets have lower BMD (55–58). The optimal intake of sodium for calcium conservation and to meet the American Heart Association (AHA) guideline is 2400 mg per day. An adequate intake of calcium allows a more liberal use of sodium in the diet. Recently, the Dietary Approaches to Stop Hypertension (DASH) diet was shown to reduce bone turnover (39). Increased sodium intake leads to increased renal calcium excretion. However, if AHA guidelines are followed (2400 mg sodium/d) there will be no negative impact on bone health. Markers of bone resorption do relate to sodium intake but generally BMD does not relate to sodium intake. The anion is important with sodium chloride increasing urinary calcium more than other salts such as sodium bicarbonate or sodium acetate (60). Sodium intake will not be a problem in the face of adequate calcium intake (61) or potassium (55).

POTASSIUM

The main importance of potassium is based on the influence of potassium on calcium homeostasis, particularly the urinary conservation and excretion of calcium. Low potassium diets increase urinary calcium losses and high potassium diets reduce it. Potassium is found in several vegetables, fruits, legumes, and milk and tends to have alkaline ash characteristics. There have been some studies relating the Net Endogenous Acid Production (NEAP) to potassium intake and bone density (62–63). Furthermore, increased intake of potassium citrate was able to ameliorate the higher bone resorption seen with high salt diets (55). Higher potassium intake, primarily from fruits and vegetables, was associated with higher baseline BMD and less bone loss (64). The need to ensure adequate potassium intake from fruits and vegetables is a strong rationale for the “5 to 10 servings per day recommendation” (65).

VITAMIN K

Vitamin K is a fat-soluble vitamin that functions as a cofactor in enzymes involved in the synthesis of blood coagulation factors and may be required for bone metabolism, to facilitate carboxylation of proteins such as osteocalcin (involved in bone formation) and to reduce urinary calcium excretion (66–67). Vitamin K is present in dark green leafy vegetables, fruits, and vegetable oils with small amounts in dairy products and grains. Vitamin K₂ is found in fermented dairy and soy products, fish, meat, liver, and egg. The current adequate intake (AI) for vitamin K is set at 120 µg for men and 90 µg for women.

Observational studies indicate that vitamin K intake and serum levels are positively related to bone density (68–71) and patients who sustain fractures have been reported to have lower serum vitamin K levels. Epidemiologic studies have also found that higher vitamin K intake is related to lower fracture incidence (72–75). Furthermore, a high percentage of undercarboxylated serum osteocalcin as seen with low serum vitamin K may be a predictor of fracture risk (66, 76–78), although many of these studies are confounded by overall poor nutrition. However, in healthy girls with a typical US diet, better vitamin K status was associated with decreased bone turnover (79).

Deficiency or antagonism of vitamin K (coumarin derivatives) can result in the undercarboxylation of specific proteins involved in bone metabolism including osteocalcin. In cohort studies, warfarin use for more than 1 year was an independent predictor of spine and hip fracture (80) but this was not confirmed in a separate study (81), perhaps because of the small number of women on warfarin.

Several small controlled vitamin K supplementation studies have found reductions in calcium excretion, bone resorption, and the undercarboxylated fraction of osteocalcin. A compound derived from vitamin K (MK4) had a positive effect on BMD in large doses when given to women with osteoporosis (82) and strokes (83). High, pharmacologic doses of vitamin K2 (45 mg) were also related to lower rates of bone loss and a lower incidence of fractures (84–85).

Based on the current evidence of observational studies, studies on intermediate endpoints, and small studies with bone density and limited fracture data, there are insufficient data to recommend the required level of vitamin K supplementation for optimal bone health. One trial with a 3-y supplementation of phyloquinone (1 mg/d) with calcium and vitamin D reduced hip bone loss (86). A healthy diet, high in fruits and vegetables, ensures that vitamin K intake is adequate for most of the population.

VITAMIN C

Vitamin C is an essential cofactor for collagen formation and synthesis of hydroxyproline and hydroxylysine. Rich dietary sources of vitamin C include citrus fruit and juices, peppers, broccoli, and tomato products and green leafy vegetables. The dietary reference intakes (DRIs) for vitamin C are 75 mg/d for adult women and 90 mg/d for adult men. Epidemiologic studies show a positive association between vitamin C and bone mass; low intakes of vitamin C are associated with a faster rate of BMD loss, and one study found that higher vitamin C was associated with fewer fractures; however, there are no randomized clinical trials (87–94). Recommended intakes of 5 or more servings of fruits and vegetables should supply enough vitamin C for bone health.

VITAMIN A

Recommended dietary allowance of vitamin A is 800 µg/d retinol equivalent (RE) for females and 1000 µg/d RE for males. Vitamin A is a fat-soluble vitamin required for vision, growth, fighting infection, and for bone remodeling. There are different types of vitamin A in the diet and in supplements: retinol and β-carotene (and other carotenoids). Excess vitamin A may be detrimental to bone health with intakes of higher than 1500 µg of RE related to a 2-fold increased risk of hip fracture in the United States and Sweden but not in Iceland or in another US study (95–98). These population studies show excess vitamin A intake from retinol appeared to increase the risk of hip fracture.

There is no evidence of any association between β-carotene intake and osteoporosis or related fracture. Vitamin A from fruits and vegetables (carotenoids) does not negatively affect bone health.
MAGNESIUM

Magnesium, complexed with adenosine triphosphate (ATP) takes part in many enzyme reactions including synthesis of proteins and nucleic acid. The intake recommended for healthy adult males is 420 mg/d and for women is 320 mg/d. Because magnesium is present in most foods—particularly legumes, vegetables, nuts, seeds, fruits, grains, fish and dairy—severe magnesium deficiency is rarely seen in healthy people. However, many intakes in the United States fall below this recommended level. Furthermore, a magnesium supplement may be required in frail elderly with poor diets (99) or persons with intestinal disease (100), alcoholics, or persons on treatment with diuretics or chemotherapy that depletes magnesium. In addition, as calcium supplements sometimes result in constipation, a supplement with magnesium might be useful to keep bowel habits regular.

Magnesium deficiency is easily detected with biochemical symptoms (eg, low serum magnesium, low serum calcium, resistance to vitamin D) or clinical symptoms (eg, muscle twitching, muscle cramps, high blood pressure, irregular heartbeat). Lastly, magnesium deficiency is easily treated.

Several small epidemiologic studies have found that higher magnesium intakes are associated with higher BMD in elderly men and women (101). There have been only small controlled clinical trials of magnesium supplementation (102–103) that were primarily effective in magnesium-depleted subjects. There is little evidence that magnesium is needed to prevent osteoporosis in the general population. Overall, observational and clinical trial data concerning magnesium and bone density or fractures are inconclusive and, in fact, one recent study from the WHI reported that higher intakes of magnesium were associated with a higher risk of wrist fracture (104).

FLUORIDE

Fluoride is an essential trace element that is required for skeletal and dental development. The adequate daily adult intake is 4 mg for males and 3 mg for females. The concentration of fluoride in the soil, water, and many foods varies by geographic region. Major dietary sources include drinking water, tea, coffee, rice, soybeans, spinach, onions, and lettuce. There is no need to add fluoride supplements to an adult diet for skeletal health. The lower doses of fluoride typically found in drinking water have no effect on bone density or on fractures (105–107); however, in some endemic high fluoride areas, higher hip fracture rates have been seen. Excess fluoride ingestion causes fluorosis, a painful condition associated with extra-osseous calcification and brittle bones. High doses of fluoride can stimulate osteoblasts; however, the quality of bone that is formed may be abnormal and the effect on fracture rates is unclear (108).

OTHER NUTRIENTS

The effects of trace metals on bone remain unknown. Three studies have shown that a combination of several minerals (zinc, manganese and copper) with calcium was able to reduce spinal bone loss in postmenopausal women (109–110).

Boron is not an essential nutrient so there are no recommended intakes. Although studies have found that 3 mg daily of boron may have a positive effect on bone (102, 111), controlled trials are needed. Boron is present in several foods such as fruits, vegetable, nuts, eggs, wine, and dried foods. Copper is an essential element required by many enzymes including lysyl oxidase, which is required for cross linking of collagen. Severe deficiency does have profound effects on bone. There have only been a few intervention trials with variable results on bone turnover and bone density (112–114), or a mixture of trace elements has been studied (109). Profound zinc deficiency leads to reduced bone growth and maturation. However, there is little evidence that zinc has an effect on bone mass or osteoporotic fractures.

Dietary silicon intake was reported to correlate with BMD at the hip in a cohort of men and premenopausal women (115). These results will require further follow-up.

In 2 recent studies, low vitamin B12 status was associated with low BMD in men and women, and osteoporosis in elderly women but not men (116,117). It is unclear whether associations such as this are really an indication of overall poor nutrition and frailty. Similarly, in another study increased dietary iron intake was associated with greater bone mineral density at all sites (118).

RECOMMENDATIONS

The nutritional needs for optimizing bone health can be easily met by a healthy diet with adequate calcium and vitamin D intakes through dairy or calcium fortified foods. Foods are a preferred source to maintain calcium balance because there are other essential nutrients that are found in high calcium foods. For those individuals where there is inadequate calcium intake from diet, supplemental calcium can be used. Supplemental or dietary calcium should be spread out throughout the day with 500 mg or less being consumed at each meal to optimize absorption.

In all individuals over the age of 70, vitamin D intakes of at least 600 IU per day (ideally 800-1000 IU/d) are recommended, in addition to the calcium requirement of 1200 mg/d. Vitamin D from foods, supplements, and/or multivitamins can be used to meet the vitamin D requirement. Recent evidence suggests that the optimal level of serum 25(OH)D may be close to 80 nmol/L (7, 8). Severe vitamin D deficiency can be easily treated by giving the patient an oral dose of 50 000 IU of vitamin D once a week for 8 wk or by giving 50 000 IU of vitamin D daily for 10 days (119–120). The fortification of food products is becoming frequently used as a method to improve calcium intake and may also be a reasonable method to increase the vitamin D intake of the population and reduce the prevalence of hypovitaminosis D. The use of calcium and vitamin D supplements in an elderly population has been shown to be cost-effective for hip fracture prevention (121–122). Medical professionals need to be aware of the importance of ensuring adequate calcium and vitamin D intakes for patients on osteoporosis therapy (123).

The effects of calcium and vitamin D on bone cannot be considered in isolation from the other components of the diet (124). The other micronutrient needs for optimizing bone health can be easily met by a healthy diet that is high in fruits and vegetables (5 servings per day) for magnesium, potassium, vitamin C, vitamin K and other potentially important nutrients (125–126).

REFERENCES


Micronutrient requirements in older women\textsuperscript{1–3}

Ronni Chernoff

ABSTRACT
The nutritional requirements of older women is an area of great interest because the extended life expectancy leads to an increase in women living into their 80s, 90s, and longer. The recommended dietary allowances (RDAs) and dietary reference intakes (DRIs) are not specific for women living to advanced ages, and little research has been conducted specifically on the micronutrient needs of elderly women. Older adults are at greater risk for nutritional deficiencies than are younger adults due to physiologic changes associated with aging, acute and chronic illnesses, prescription and over-the-counter medications, financial and social status, and functional decline. Among the significant age-associated changes in nutrient requirements, the need for energy decreases and the requirements for protein increase with age. Among the micronutrients, the significant ones that may be associated with deficiencies in elderly women include vitamin B-12, vitamin A, vitamin C, vitamin D, calcium, iron, zinc, and other trace minerals. In old and very old women, these are micronutrients of interest but there is a great need for research to determine appropriate recommendations. The importance of these selected nutrients and the reasons for the likelihood of deficiency are discussed briefly. However, there is little specific information regarding micronutrient requirements for elderly women. One reason for this is the difficulty in conducting reliable and valid studies due to the heterogeneity of older adults and their unique rate of aging associated with their health status, limited income, disability, and living situation.

KEY WORDS
Micronutrient requirements, older women, energy, protein, vitamins, trace minerals

INTRODUCTION
The nutritional requirements of older women is an area of great interest, particularly because their life expectancy continues to increase due to better health care and earlier awareness of health promotion activities. Interestingly, though interest in the area of nutritional requirements is increasing, there is a lack of research targeted to exploring the needs of the very elderly. The nutrition recommendations provided by the RDAs and the DRIs levels are not helpful when it comes to providing advice to old and very old women, although in the most recent publications there are separate recommendations for individuals over age 70 (1–3); little research has been conducted specifically on the needs of elderly women.

Older adults are generally at greater risk for nutritional deficiencies than are younger adults. They experience the normal changes associated with aging (decrease in lean body mass, decrease in total body water, decrease in bone density, and an increase in the proportion of total body fat), as well as physiologic challenges associated with chronic and acute medical conditions. Compounding these changes, there may be environmental, social, financial, and functional barriers faced by older women that may interfere with adequate dietary intake.

One of the most significant changes that is seen in old and very old adults is a decrease in basal energy requirements; this can generally be attributed to the decrease in lean body mass. A reduction in energy expenditure is also associated with sedentary behavior and a loss of mobility related to systemic (eg, cardiovascular, pulmonary) or bone and joint disease. To avoid weight gain, which may compound already existing functional deficits (4, 5), dietary intake may be decreased. A reduction in nutrient consumption will add to a decrease in overall dietary quality because favorite foods are consumed and foods that are less well-liked (vegetables) are often the ones first eliminated. Nutrient-dense foods often fall into that category; favorite and “comfort” foods are frequently those that are high in fat and carbohydrate. Obtaining sufficient amounts of micronutrients becomes a challenge within the complex nature of the human aging process (6).

Risk for poor nutritional status is also related to a decreased efficiency of the gastrointestinal tract that occurs in some elderly people. Chewing, swallowing, digesting, and absorbing nutrients may be impaired for a variety of reasons (7). Oral health status, edentulousness, dentures that may not fit properly, or lesions in the oral cavity will interfere with consumption of a well-balanced diet and sufficient intake to meet nutrient needs (8). Nutrients may not be as proficiently digested and absorbed due to atrophic gastritis, a decrease in hormone and enzyme production, senescent changes in the cells of the bowel surface, and the interactions among drugs and nutrients (7). Constipation is a chronic problem in many older adults; this may be associated with a decrease in peristaltic strength or in adequate dietary fluids and fiber to provide bulk.

Compounding these changes is the effect of both chronic and acute illness (6). Dietary modifications are frequently introduced...
to accommodate loss of functional capacity to feed oneself, effectively lower sodium intake, manage lipid profiles, control blood glucose levels, lose weight, or manage other metabolic conditions associated with disease. Demands for specific nutrients may be increased because of increased needs associated with healing, recovery, or rehabilitation (9). Dietary intake in elderly women may decrease due to alterations in their health, functional or cognitive status, disease-related anorexia, or changes in taste sensitivity often associated with medication use.

Many very old women face challenges associated with their environment, social and financial status, and their level of functional ability. Many older women have been widowed, have had their children move to other geographical areas, are living on a fixed income, and experience disability. Cooking for one may not be an activity that motivates an elderly woman after years of shopping and preparing food and meals for a family and spouse. There may be financial challenges associated with limited income, living on pensions, or social security. Health care costs can be burdensome, even for someone in relatively good health; costs for someone who has multiple chronic conditions or acute illness may be financially disastrous. Because older women have a greater life expectancy than elderly men, there is a greater likelihood that they will experience more disability in their later years simply because they live longer. Disability may lead to elderly women becoming homebound or more dependent on social services that will help them meet their basic needs. Obesity is a risk factor for disability, and sedentary lifestyle is a risk factor for obesity; this is a difficult cycle to avoid but one that adds burden to nutritional intake and status (4–6).

**NUTRIENT NEEDS OF VERY OLD WOMEN**

Although there is little research conducted on micronutrient requirements in elderly women, there are key nutrients that demand attention. Though not a micronutrient, protein is an important nutrient for old and very old individuals. Protein needs actually increase with age (10,11). Because lean body mass decreases with age, it would seem that protein requirements would decline, but they increase to maintain nitrogen equilibrium; when demands increase to heal wounds, fight infection, repair fractures, or restore muscle mass lost from immobility, dietary protein must be increased above maintenance requirements but frequently protein is overlooked as a target nutrient in the very old patient.

Among the micronutrients, the significant ones that may be associated with deficiencies in elderly women include vitamin B-12, vitamin A, vitamin C, vitamin D, calcium, iron, zinc, and other trace minerals. In old and very old women, these are the micronutrients of interest and there is a need for a great deal more research.

**Vitamin B-12**

Vitamin B-12 is a nutrient of interest in the old and very old woman primarily because the consumption of foods rich in this nutrient decreases with age (12). (Figure 1) The bioavailability of protein-bound vitamin B-12 decreases as people age. The mechanism that is most affected by age is the ability to cleave the vitamin from its protein carrier; the prevalence of atrophic gastritis, reported to be 40%–50% of individuals over age 80, is a severe impediment to the transport and release of vitamin B-12 (13). The production of gastric acid is necessary for the digestion of food rich in vitamin B-12. Animal protein, the primary source of vitamin B-12, is expensive, difficult to chew, and has been associated with elevated blood lipids. Bacterial overgrowth in the gut may also be a factor in the reduction in the bioavailability of vitamin B-12; bacteria may bind the B-12, rendering it unavailable for absorption (14). This condition is easily treated with antibiotics.

Vitamin B-12 requirements that are not met through diet can be met with supplements that contain crystalline vitamin B-12; however, there is still a limited bioavailability (15). For elderly adults, the recommendation to meet vitamin B-12 needs is through foods fortified with B-12 or B-12-containing supplements.

**Vitamin A**

Vitamin A has many roles in the maintenance of health; it is important to maintain normal vision, for cell differentiation, efficient immune function, and genetic expression (6, 16). Vitamin A recommendations for older adults have been lowered from previous editions of the RDAs (2). Present suggested levels are 700 μg retinol activity equivalents (RAEs) for women and 900 μg RAE for men. Some researchers have recommended that these recommendations be set at even lower levels because although the vitamin A intake for many older adults is below current recommendations, their vitamin A levels remain normal (17). (Figure 2) It has been suggested that dietary vitamin A be obtained from an increased intake of carotenoids, including β-carotene, lycopene, zeaxanthine, and lutein, among others (18).

It is not common to find vitamin A deficiency in elderly individuals in the United States, as vitamin A is easily obtained from food as well as dietary supplements. However, absorption in
elderly adults increases, therefore making the possibility of toxicity greater if supplements with high levels of vitamin A are included in the diet daily. Compromised hepatic function may contribute to an increased risk of toxicity, particularly in those who are using supplements or eating fortified foods. In old adults who may have asymptomatic hepatic dysfunction, the risk for vitamin A toxicity increases. Levels of retinyl esters rise when liver damage or vitamin A toxicity occurs (19).

One consequence of high vitamin A intake is its association with a higher risk for fractures. Vitamin A is a vitamin D and calcium antagonist and a high intake of vitamin A over long periods of time may create serious bone health problems (20). Obtaining supplemental vitamin A in its precursor form, β-carotene, appears to be considerably safer, more effective, and has not been associated with adverse or unanticipated side effects. Consuming a diet rich in fruits and vegetables is a reasonable way to meet vitamin A needs in older adults as well as providing a good source of dietary fiber.

Vitamin C

Vitamin C status is generally related to dietary intake; presently, requirements for older adults are higher than the 1989 RDAs. Newer data set the recommendations for vitamin C at 90 mg/d for males and 75 mg/d for females over age 50 (21). Reductions in vitamin C intake are associated with illness, hospitalization, and institutionalization. Lowered intake often is associated with chronic disease including atherosclerosis, cancer, senile cataracts, lung diseases, cognitive decline, and organ degenerative diseases (21).

Vitamin C is relatively easy to replenish by consuming fruits, fruit juice, and vegetables, or through vitamin supplementation. Elderly individuals who smoke may require double the recommended intake just to maintain tissue levels. As a key nutrient, it is important to consume adequate amounts, particularly in old adults. The role of vitamin C is that of an antioxidant; as a metabolic reducing agent; as a catalyst needed for hydroxylation for proline and lysine, needed for collagen production essential to make new tissue and heal wounds; and, for the maintenance of vascular integrity.

Tissue saturation of vitamin C is achieved easily (22); excess dietary vitamin C will be excreted in the urine. Chronic large doses may contribute to diarrhea or renal calculi formation and should be discouraged among elderly persons.

Vitamin D

It is well recognized that older women are at risk for inadequate vitamin D consumption. Vitamin D has significant roles in bone health by regulating bone mass, but it also is an essential nutrient in immune function (9, 23). There are 2 primary sources of vitamin D: diet and skin. Dietary sources of vitamin D are fatty fishes and fortified dairy products. Consumption of fortified dairy products is very variable among older women, especially because lactose intolerance is more prevalent in older adults. There has been some speculation that even in people who are including fortified foods in their diet, the foods are underfortified (6).

Skin as a source for vitamin D precursor may be helpful for those who live in temperate climates where they can get outside daily, however, during the winter months many elderly people do not get out at all if they live where it is cold (6). For those who live in warmer areas, fear of skin cancer from too much sun exposure is also an impediment to the activation of vitamin D precursors. Additionally, the vitamin D precursor found in skin decreases with age. The ability of the kidney and liver to hydroxylate vitamin D precursors is affected by age, thereby suggesting that the vitamin D requirements might be higher than have been recommended (24).

Calcium

Calcium is an essential nutrient that many older women consume in inadequate amounts. (Figure 3) Despite this, the endocrine system serves to maintain serum calcium within a fairly narrow range by managing absorption, bone mineral balance, and calcium excretion in urine (25). For older women, the dynamics of calcium requirements change. Presently the DRI for calcium for adult women is 1200 mg calcium/d but there have been suggestions that a daily intake of 1500 mg/d for postmenopausal or over 65-year-old women would be appropriate (26). The challenge is that when the RDA was 1000 mg/d there were a significant number of women who consumed diets with inadequate levels of calcium; changing the requirement does not necessarily alter eating patterns and it is apparent that supplemental calcium is needed from nondietary sources.

Calcium has been linked to the prevention or lowered risk for many chronic conditions such as osteoporosis (27, 28), colon cancer (29), and hypertension (30).

Iron

Requirements for iron for women change after menopause; the need for iron to replace menstrual losses ceases, and tissue stores are generally adequate if dietary intake is at acceptable levels. Needs for iron in older women revert to the same levels as those for adult males: 10 mg/d. Dietary iron bioavailability may be affected by the consumption of heme iron, supplemental iron, dietary ascorbic acid, and alcohol (31, 32).

If there is an inadequate consumption of dietary iron, iron deficiency anemia may occur. In older adults, this would indicate a significant deficiency over a long time. However, there is little evidence that iron deficiency is prevalent in either an American or European population of older adults (33). Iron deficiency anemia is most often encountered in older adults who have comorbidities, particularly chronic inflammatory diseases. Iron deficiency anemia can be diagnosed using serum ferritin measures, plasma transferrin receptors, and erythrocyte sedimentation rates. There is an inverse relation between tissue iron stores and serum ferritin levels.

![FIGURE 3. Mean intake of calcium (mg) in adults age 20–80+, NHANES III, Ref 12.](Image 320x78/L1151)
Although iron deficiency may not be the most common type of anemia seen in older adults, it is seen among hospitalized, institutionalized, or chronically ill older adults. Usually, anemia associated with age is due to chronic bleeding in the gastrointestinal tract. Iron deficiency anemia may be manifested as “restless legs syndrome” (34); this condition is easily treated by providing iron supplementation. To enhance absorption, iron should be taken in divided doses and with meals, which will decrease the gastrointestinal side effects. If oral supplementation is not tolerated, parenteral iron may be given as a short-term intervention.

Zinc

Zinc has been recognized as an essential trace nutrient since the 1930s. It is a metal that has a role in many enzymes, in gene expression, and in immune function, among other physiologic functions (35). Marginal intake of dietary zinc will lead to a lower physiologic zinc levels, but the real challenge may be factors that inhibit or interfere with zinc absorption. Divalent positively charged ions, such as iron, can interfere with zinc bioavailability. Phytates, from grains, cereals, rice and legumes, may interfere with the absorption of zinc; the zinc found in vegetable foods may be less bioavailable than zinc from animal sources (35).

Malabsorption, physiologic stress, trauma, and muscle wasting will all contribute to inadequate zinc status, as will both prescription and over-the-counter medications. For older women, these circumstances may occur too frequently. Consequences of poor zinc status may include reduced immune function, dermatitis, loss of taste acuity, and impaired wound healing (35).

In zinc deficient elderly individuals, T lymphocyte impairment and cellular immunity are compromised (36, 37). Zinc replacement will improve immune status. Inadequate zinc status will affect the efficiency of wound healing, and zinc supplementation will contribute to more efficient healing; however, zinc supplementation in individuals with adequate zinc nutriture will not produce any benefit.

Dermatitis is a commonly seen manifestation of zinc deficiency, as is a decreased sense of taste. Zinc supplementation may contribute to a reversal of symptoms in individuals who are zinc deficient. In individuals who have adequate zinc status, supplementation will not improve their conditions. In fact, too much zinc may lead to suboptimal absorption of other trace minerals (38).

Copper

Copper is a positively charged divalent ion that is an essential nutrient. Large doses of zinc will interfere with the absorption of copper. Copper deficiency is difficult to diagnose and is not often thought about. Symptoms are vague and can easily be associated with other nutritional or medical problems. In humans, copper deficiency may manifest as hypochromic anemia, osteoporosis, arterial disease, myocardial symptoms, and decreased metabolic activity of copper-containing enzymes. Copper deficiency has been linked with hypercholesterolemia, glucose intolerance, and hypertension, all of which are not unusual to see in elderly adults (2, 39).

Copper is widely distributed in a variety of foods and is relatively accessible if a diet with variety is consumed in adequate amounts. Estimated average requirements (EAR) for copper for adults to age 70 have been established at 700 μg/d (2). The RDA is 900 μg/d.

OTHER TRACE NUTRIENTS

Chromium

Chromium status in adults is most usually related to dietary intake. Impaired chromium absorption is generally associated with high intake of nutrients that impact on bioavailability or that increase excretion; these food components include high levels of fiber or simple sugars that increase urinary excretion. In individuals who have low levels of chromium, symptoms of hyperglycemia or abnormal lipid levels may be corrected by providing supplementation designed to normalize chromium status (39, 40).

Selenium

Selenium has been identified as an essential nutrient; however, it is rare to see selenium deficiency in the United States (21). However, selenium deficiencies have been reported among institutionalized elderly, particularly those who have multiple pathologies (41), but, once identified, this is easily corrected with supplementation. Selenium does function as an antioxidant and individuals may take supplements to enhance immune function or increase antioxidant activity. There is an upper limit of 400 μg of selenium/d, which, if exceeded, may lead to toxicity. Symptoms of toxicity may include nausea, vomiting, hair loss, irritability, peripheral neuropathy, and fatigue.

Aluminum

Although aluminum has not been identified as an essential nutrient, it is worthy of mention due to its past association with the development of Alzheimer’s disease (42, 43). Elderly women may have exposure to aluminum through excessive use of antacids, buffered analgesics, some antulcer compounds, and other medications (37). Although absorption of exogenous aluminum may increase with genetic predisposition, advancing age or mucosal damage, aluminum is absorbed in relatively small amounts. Since Alzheimer’s disease was first described in 1907, the association between dietary aluminum, use of over-the-counter medications, use of aluminum cookware, or dialysis fluids that contain high amounts of aluminum is extremely unlikely.

Magnesium

Recommendations for intake of magnesium is 350 mg/d for men and 280 mg/d for women, and there is no indication that elderly adults have needs different from younger adults (25). Intakes of magnesium in elderly people may be marginal but, even in individuals with atrophic gastritis, there does not appear to be any interference with magnesium absorption. However, hypomagnesemia should be considered as a possible factor in depressed immune function, muscle atrophy, osteoporosis, hyperglycemia, hyperlipidemia, and other neuromuscular, cardiovascular, or renal dysfunction (25).
Boron

Boron is a trace mineral that may have some importance for elderly women due to its association with calcium metabolism and bone mineralization (44). Whether boron will be assigned a recommendation or requirement is not presently clear, but high levels of boron in association with magnesium may lead to increased excretion of calcium (45).

Lead

Lead may not be an essential nutrient and is most closely linked with problems in growth and development in children in poverty, particularly in housing built and painted with lead-based paint years ago. However, lead poisoning from consuming lead-based paint has been reported in elderly, institutionalized individuals (46).

SUMMARY

There is little specific information regarding micronutrient requirements for elderly women. As the population grows older, the need for more information on nutrient needs in very old people will become more important; one of the challenges in defining nutritional needs is the heterogeneity of elderly adults, compounded by the likelihood of multiple chronic conditions, use of many prescription and over-the-counter medications, and the variable quality of nutritional intake associated with limited income, disability, and institutionalization. It seems that the metabolic changes that occur with aging would have some impact on vitamin, mineral, and trace element needs, but there is a clear need for future research to elucidate these nutrient needs.

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Micronutrient requirements of physically active women: what can we learn from iron? 

Sharon R Akabas and Karen R Dolins

ABSTRACT

The health benefits of physical activity are well established and there is increasing recognition of the importance of fitness as a key modulator of chronic disease. The impact of physical activity on micronutrient requirements is a topic of tremendous interest to the lay public, but the interest is in sharp contrast to data from well-designed studies. Research in this area is poorly controlled for nutritional status of the participants, standardized exercise protocols, markers and cutoff points for measurement of micronutrient status, and variability in subject characteristics. The micronutrient status of women in the general population is of concern, but it is not clear that physical activity increases the requirement of most micronutrients. When dietary intake is adequate, the results of most studies are either equivocal or show no benefit to performance of supplementation. In the few instances where exercise does appear to increase an individual’s requirement, the increase can be obtained within the additional calories required for energy balance. In the absence of consistent data, micronutrient supplementation is often indiscriminate without regard to nutrient status. Because iron is such a key nutrient for physical activity, and the status in women is often compromised, it serves as a useful example of how current research limits the ability to make recommendations regarding the impact of exercise on micronutrient requirements in women. With the recent recognition of the importance of physical activity to the prevention and treatment of chronic diseases through the life span, more attention should be focused on the impact of exercise on micronutrient requirements, especially in the context of weight loss regimens. Am J Clin Nutr 2005;81(suppl):1246S–51S.

KEY WORDS Exercise, physical activity, iron, micronutrients, women, supplementation, nutritional status, dietary intake, exercise protocol, markers

INTRODUCTION

Though the health benefits of physical activity are indisputable (1–8), its impact on micronutrient requirements is much less well established. In the case of many micronutrients, women have inadequate intake (9), and a deficiency has a known detrimental impact on physical performance. Less clear is whether physical training and physical activity increase the requirement of particular micronutrients, and whether amounts in excess of the dietary reference intakes (DRIs) should be recommended for physically active individuals. In the few instances where exercise does appear to increase an individual’s requirement, the increase can be obtained within the additional calories required for energy balance (10–12). A large gap on micronutrient requirements exists in the literature regarding the impact of a weight loss regimen’s that include exercise.

Much of the uncertainty regarding micronutrient requirements and exercise stems from the lack of standardization or assessment of the nutritional status of the participants at entry, failure to assess subject’s dietary intake, lack of standardization of the exercise protocol or program, lack of standardization of markers and cutoff points, difficulty comparing one subject population (sex, menstrual status, age) to another, and failure to differentiate between nutritional requirements during the initial training period and those of the period that follows the adaptive phase.

In the absence of clear recommendations, micronutrient supplementation is often indiscriminate without regard to nutrient status, due to the belief that it cannot hurt and may help. This assumption is erroneous. In some instances, additional micronutrient ingestion beyond the DRI not only has no effect on exercise performance (12-14) but may have negative impact on general health (15, 16).

Many of these issues have been comprehensively addressed in recent reviews of the topic of exercise and micronutrients (13, 14, 17). This article uses iron as an example of how the limitations in current research constrain the ability to make clear recommendations regarding the impact of exercise on micronutrients requirements in women. The article concludes with the practical implications of the ambiguity, and suggestions for future research.

BRIEF OVERVIEW OF IRON PHYSIOLOGY AND REQUIREMENTS

Iron is a critical nutrient for active individuals, male and female alike. It plays a key role in energy production as a carrier of oxygen, both in the form of hemoglobin in the blood and myoglobin in the muscles. Additionally, iron is a part of the cytochromes found in the electron transport system.

The recommended dietary allowance (RDA) for premenopausal women is 18 mg daily. While additional iron is recommended for pregnancy and lactation, an increased intake is not

1 From the Institute of Human Nutrition (SRA) Teacher’s College (KRD), Columbia University, New York.
2 Address reprint requests and correspondence to SR Akabas, Institute of Human Nutrition, Columbia University, 630 West 168 Street, PH 1512-East, New York, NY 10032. E-mail: sa109@columbia.edu.
3 Address reprint requests and correspondence to SR Akabas, Institute of Human Nutrition, Columbia University, 630 West 168 Street, PH 1512-East, New York, NY 10032. E-mail: sa109@columbia.edu.
concentrations below 12 mg/dL in males. Menstruating women carry an increased risk for iron deficiency regardless of training status due to monthly blood loss. Adding to this concern, survey data shows that female athletes often underconsume calories (19–21), making it less likely that they will consume adequate dietary iron.

As iron clearly plays a critical role in athletic performance and deficiencies are not uncommon, female athletes are often advised to supplement iron without prior determination of hematological parameters. This practice is discouraged by medical practitioners due to the danger of toxicity from iron overload.

Iron depletion occurs in 3 stages. The first stage, depletion of stores, is identified by serum ferritin below 12 μg/L. The second stage, iron-deficient erythropoiesis, is identified by increased concentrations of transferrin and reduced transferrin saturation. Stage 3, anemia, is characterized by microcytic hypochromic red blood cells and diagnosed as hemoglobin below 12 mg/dL in females and below 13 mg/dL in males.

While it is clear that iron deficiency anemia impairs athletic performance, it is less clear whether iron deficiency without outright anemia will have this effect. Thus a focus of current research is whether iron deficiency without anemia will impair performance and, if so, whether and at what point iron supplementation should be initiated.

The choice of markers of iron deficiency has complicated clarification of this issue. Serum ferritin, the most commonly used marker, may become elevated in response to inflammation, infection, liver disorders, malignancies, and exercise-induced hemolysis (22). Iron deficiency may therefore be masked in certain individuals. Also clouding this issue is the lack of standardization in cutoffs used for diagnosis. Though serum ferritin concentrations below 12 μg/L is typically considered to be the marker for depletion of stores, researchers have use varying levels in developing inclusion criteria for their studies.

Measurement of serum levels of soluble transferrin receptor (sTfR) has recently become the gold standard for identifying iron deficiency in its earliest stage. First described in 1963, sTfR is bound to the cell membrane and mediates the endocytic transfer of iron from transferrin into erythroid cells. Levels increase when iron stores are depleted or turnover stimulated. Small amounts appear in the blood and can be measured. This marker, therefore, reflects both iron stores and the rate of erythropoiesis, and has been found to be more sensitive to iron deficiency than serum ferritin as well as more indicative of the functional pool of iron (23). At this time, this marker is the most accurate indicator of iron stores, and least confounded by factors such as inflammation (24). Values may be confounded by muscle growth, as this will result in a rise in sTfR levels due to a need for increased erythropoiesis. Thus muscle growth must be taken into account during analysis of changes in sTfR. In addition, the most appropriate cutoff point must be clearly established to allow a meaningful consensus to emerge regarding physical activity and iron requirements.

DOES EXERCISE INCREASE IRON REQUIREMENTS?

Proposed mechanisms for an increased requirement for iron include increased losses in sweat, feces and urine, intravascular hemolysis, and impaired absorption. Endurance athletes are known to experience sports anemia, a dilution of ferritin and hemoglobin due to the plasma volume expansion that occurs with training. This is a transient effect that occurs when plasma volume increases more rapidly than the increase in red blood cell mass, and typically results in a dilution of ferritin and hemoglobin of 15% (25).

Long distance runners may experience greater gastrointestinal losses through the feces. Though female data have not been provided, Telford and colleagues (26) studied hemolysis in male triathletes after a 1-hr cycle and run. They found that plasma free hemoglobin and serum haptoglobin concentrations were increased after both exercise bouts, but the increase was 4 times greater after the run than after the cycle. The authors concluded that footstrike is the major contributor to hemolysis during running.

Iron losses may occur through sweat and desquamated epidermal cells. One early study conducted in healthy males, in which the skin was carefully cleaned to remove desquamated epithelial cells and thus isolate sweat, found sweat iron losses to be small (22 μg per liter sweat) and thus unlikely to have an impact on iron requirements (27). A more recent study of female athletes found that sweat losses of iron declined with time (28). The greatest concentration of iron in sweat occurred during the first 30 min of exercise, and was lower in a hot environment than a neutral environment. Sweat iron concentration in this study was related to ferritin concentration, suggesting that conservation of iron may occur with reduced stores. The authors estimated that 5.7% of daily absorbed iron, or 1.2 mg/dL, would be lost by exercising females during the first hour of exercise, and that this could contribute to depletion of iron stores.

The effect of exercise on iron absorption has been questioned. While female data are not provided in this area either, moderate intensity exercise did not impair iron absorption in a study on male cyclists exercising at 60% VO2max for one hour (29). To the contrary, absorption of 100 mg ferric sodium citrate led to a 48.2% increase in serum iron concentrations when taken 30 min before exercise as compared with an increase of 8.3% when taken at rest.

Based on the available research, it is difficult to arrive at conclusions regarding the impact of exercise on iron requirements in women. If there is an increase in requirements, it will most likely be for women engaged in long distance running due to gastrointestinal losses and footstrike hemolysis. To fully clarify the confusion, future research protocols must control for diet, menstrual status, standardize exercise protocols, and use sTfR as the primary marker of iron status.

EFFECT OF TRAINING ON IRON STATUS

Strength training

Numerous studies have looked at changes in iron status that occur with training. In a study designed to examine the effects of resistance training on iron status, Murray-Kolb and colleagues tested 17 older, postmenopausal women (54–71 y old) and 18 men (56–69 y) before and after a 12-wk resistance training program (30). Diet including bioavailability of iron was evaluated with a 3-day food log, supplements accounted for, and compliance monitored. All hematological measures, including iron, transferrin, ferritin, and sTfR were within normal limits for both
genders at baseline and after training. However, women did experience a significant decrease in ferritin and a trend toward increased TIBC, whereas men experienced a rise in sTfR, probably related to an increase in lean mass, which was not achieved by the women. Thus the researchers found that men and women experienced sex-specific changes in iron status, and noted that a rise in sTfR during resistance training may indicate muscle growth rather than iron deficiency. Deruisseau et al (31) designed a similar study with collegiate men and women participating in a 12-wk weight training program. Diet and adherence were monitored. Contrary to the previous study, only the men experienced a decline in ferritin, and no change in sTfR was observed in subjects of either sex.

Despite the care taken by both research teams to control for diet, and to use state of art assessment of iron status, these 2 studies fail to resolve the question of whether strength training increase iron requirements, perhaps due to differences in exercise protocols. Clarification is needed regarding whether the observed rise in sTfR during resistance training indicates muscle growth or iron deficiency.

Endurance training

The iron status of female triathletes was assessed before and after a competition consisting of a 1.5 km swim, 40 km cycle, and 10 km run (32). Of the 12 athletes studied, 2 presented with anemia based on hemoglobin below 12 mg/dL and 4 met the researcher’s criteria for iron deficiency of ferritin below 10 μg/L before the event. Serum transferrin receptor was elevated in one subject before the race. After the race, ferritin concentration remained elevated after correction for hemoconcentration, while sTfR levels did not change. The dietary intake of iron in these athletes was not assessed, leaving it unclear whether iron deficiency and anemia occurred as a result of increased requirements or inadequate intake.

Ashenden et al (33) conducted a retrospective review of hematological status from 6 y of data on female rowers, basketball players, and netball players to establish changes that occur through training seasons and how the mode of training might effect those changes. Mean serum ferritin concentrations for all athletes experienced a decline in serum ferritin of about 25% during the training season. While the means did not drop below normal at any point, a sub-group presented with low concentrations (7.5 ± 2.7 μg/L). Rowers (non-weight-bearing) maintained higher levels than basketball and netball players (weight-bearing) throughout the season. Again, dietary intake was not evaluated and sTfR was not measured.

Based on these few studies, one of which focused on an acute exercise bout, the other on long-term training, it is once again too difficult to arrive at a conclusion that could serve as the basis for a recommendation for or against iron supplementation in response to training. A major obstacle to arriving at a conclusion is the failure of each study to assess dietary intake and correlate with hematological changes.

EFFECT OF IRON SUPPLEMENTATION ON NONANEMIC, IRON-DEFICIENT WOMEN

Earlier studies supplementing iron in non-anemic women have been equivocal. Reasons for this include lack of control of iron status and dietary intake, variability in subject characteristics (trained versus untrained), exercise protocols, parameters used for cutoffs, and use of different markers for measuring iron depletion.

A series of studies from the lab of Brownlie and colleagues tested the hypothesis that iron supplementation would help deficient but non-anemic women progress through a training program by improving endurance capacity (34–36). Subjects in these studies were 18- to 33-y-old untrained but active women who presented with normal hemoglobin (>120 mg/L) and low serum ferritin based on a cutoff of 16 μg/L. Subjects were given 8 mg elemental iron BID (35, 36) or 10 mg BIO (34) versus placebo for 6 wk. A 4-wk training protocol was carried out using cycle ergometers. The studies were all carefully controlled for diet using 4-d food logs and compliance with the protocol was monitored.

In the first of this series (34), subjects in the iron-supplemented group experienced an increase in serum ferritin, iron, and transferrin saturation, while sTfR concentration decreased. The placebo group was without significant changes in these parameters.

Endurance capacity and exercise performance were tested with a 15-km time trial at a level of resistance consistent with 70% VO2 max. While both groups improved their finish time in a time trial after the training program, the supplemented group was found to improve twice as much as the placebo group. Further analysis demonstrated that the effect of supplementation on endurance capacity was most pronounced in those who exhibited higher sTfR levels at baseline, suggesting an increased efficiency of oxygen utilization at the tissue level.

A subsequent study was conducted by the same laboratory (35) to further elucidate the effect iron depletion without anemia would have on women’s ability to improve aerobic capacity during a 4-week training program. The exercise protocol was the same as the previous study. Here, however, though serum ferritin, iron, and transferrin saturation increased in the supplement group sTfR did not significantly change. Exercise testing revealed greater improvements in both absolute and relative VO2 max in the supplement group as compared with placebo.

Using sTfR to further delineate iron status, results were stratified between those with initially elevated levels indicating decreased stores and erythropoiesis and those with normal levels using a cutoff of 8.0 mg/L. Those with higher baseline sTfR were found to respond the most to iron supplementation, exhibiting the greatest improvements in VO2 max. Exercise performance was not tested in this study.

In the third study of this series (36), a time trial was again introduced as a performance measure. Physiologic responses were stratified by baseline sTfR concentrations using a cutoff of 8.0 mg/L, revealing a significant effect of supplementation on % VO2max in the time trial in those subjects with baseline elevated sTfR. Improved performance in the time trial approached significance in this group compared to placebo.

The results of these studies demonstrate the value of including sTfR as a marker for functional iron deficiency. As the authors note, further research is needed to develop the most appropriate cutoff value so recommendations for supplementation can be accurately made.

Using another population, Friedmann and colleagues (37) studied the effect of iron supplementation in adolescent male and
female trained athletes with serum ferritin levels <20 μg/L and normal Hb levels. Athletes were given 100 mg elemental iron twice a day or placebo for 12 wk. A treadmill performance test was administered before and after the treatment period. Ferritin concentration increased significantly in the supplement group, while blood volume, red blood cell volume, and total body hemoglobin did not change in either group. VO2max and O2 consumption increased significantly in the iron group, although it should be noted that the increase was slight, and relative VO2max did not reach significance. sTfR was not measured, perhaps preventing the researchers from demonstrating an increase in erythropoiesis and masking a more significant improvement in a subgroup of their population. Furthermore, previous studies were successful at improving aerobic capacity and iron status with a much smaller dose of supplemental iron, leaving open the question of just how much iron is necessary to obtain the desired results.

Brutsaert and colleagues (38) used maximal voluntary contractions (MVCs) to test progressive muscle fatigae in 18- to 45-y-old women with normal hemoglobin (>110 g/L) and low ferritin (<20 μg/L) given a supplement of 10 mg elemental iron or placebo. Serum transferrin receptor, serum iron, and total iron binding capacity were measured. After a 6-wk training period, sTfR concentrations were found to rise in the placebo group, indicating a decrease in available iron. Serum iron and transferrin saturation rose in the treatment group, indicating an improvement in iron status. The rate of decline in MVCs was less rapid in the supplement group as compared with the placebo group, suggesting improved iron status at the tissue level.

Non-iron deficient adolescent male and female swimmers were studied for a 6-mo period while training for competition (39). A strength of this study was the attention paid to menstrual cycle and consequent blood loss. All subjects presented with normal levels of hemoglobin and ferritin (cutoff of normal was 7 ng/mL), but sTfR was not measured. One group was given an iron supplement of 47 mg, a second group was counseled on a high iron diet plan providing 26 mg of iron and a third group was included as a control without either intervention. Compliance with diet and supplementation was monitored. Dietary analysis showed that all groups met the RDA for iron. No performance benefit was found with supplementation in the absence of iron depletion.

Given the low cutoff used for serum ferritin, combined with the apparent adequate intake for all participants, it is not surprising that supplementation did not have an impact. In fact, this study was designed to deter unnecessary and indiscriminate supplementation that is common among female athletes.

These studies suggest that iron supplementation in nonanemic, iron-deficient women improves endurance performance. Although the range of supplemental iron was tremendous (8 mg/d to 100 mg/BID), it appears that 8 mg/d may be sufficient to achieve improvements. Three of these more recent studies (34-36) demonstrate that by controlling for dietary iron intake, reducing the variability in chosen markers, and by choosing a marker that is not confounded by inflammation, it is more likely to reach a consensus regarding physical activity and iron. The 1 study that showed no improvement is an affirmation that supplementation is only warranted in the presence of iron deficiency (39).

HIGH-RISK GROUPS

Certainly specific populations are at an increased risk for iron deficiency, including adolescents experiencing a growth spurt especially once they begin menstruating. Inadequate energy intake has been identified from numerous surveys of female athletes (20–21), which increases the likelihood of inadequate iron intake. Individuals adhering to a strict vegetarian diet are hampered by the diminished bioavailability of non-heme iron. In addition, they are likely to consume food substances that impair absorption such as phytic acid, polyphenols, calcium and phosphate salts, and soy protein, placing them at further risk. Due to these factors, the RDA for premenopausal vegetarian women is 32 mg per day, almost twice the RDA for nonvegetarians (18). In a recent review, Barr and Rideout noted that iron intake is similar among vegetarian and nonvegetarian athletes (40). The failure to meet the increased RDA for vegetarians is probably responsible for the higher incidence of functional anemia (normal Hb with low ferritin) reported in this population. Another review also concluded that a vegetarian diet may be a risk factor in iron deficiency, particularly for female runners (41).

Active women frequently remain physically active during pregnancy or may be unaware that they have become pregnant. Iron requirements increase significantly during pregnancy, and maternal anemia has been associated with an increased risk of preterm delivery (42). Therefore, it is imperative that women of childbearing ages, particular those with multiple pregnancies, monitor iron status carefully.

RECOMMENDATIONS FOR IRON SUPPLEMENTATION

The degree to which exercise itself increases iron requirements is unclear. While long-distance runners may experience gastrointestinal bleeding, it is likely that these losses will be compensated for by an increase in iron absorption (25). Sweat losses of iron do not appear to be of a degree to cause concern (27).

It is clear that iron supplementation should never be initiated without prior determination of iron status, as iron overload presents serious health issues. A group of male French elite cyclists were found in 1 study to have hyperferritinemia as a result of excessive enteral and parenteral iron supplementation (43), which may increase the risk of liver disease. Hemosiderosis is a hereditary disorder that can result in iron overload. Supplementation might lead to toxic levels in athletes with this disorder. Those who supplement need to be aware that the body does not have a mechanism by which to excrete excess iron, and that excess iron will act as a pro-oxidant, carrying with it a risk of liver cancer and cardiovascular disease. Additionally, dietary iron intake has been positively associated with the incidence of type 2 diabetes in postmenopausal women (44).

Governing groups must set protocols for evaluating iron status and initiating iron supplementation. A recent survey of NCAA Division I-A schools found that screening for iron deficiency was not routine, and that there is a wide degree of variability in the criteria used for diagnosis and treatment (45). Perhaps most interesting, hemoglobin and serum ferritin together were most often used to determine iron deficiency in the institutions responding to the survey, demonstrating that iron deficiency without anemia is not routinely identified. Serum transferrin receptor concentration is currently considered the most reliable way to
identify iron deficiency and should, in the opinion of some scientists, become the standard for assessing the iron status of athletes (16).

In an intriguing recent study of 321 early postmenopausal women (46), dietary iron was positively associated with bone mineral density in those women who had mean calcium intakes ranging from 800 to 1200 mg calcium daily. The relation remained after protein, which is also positively associated with bone mineral density, was factored out. The authors suggest that iron may play a role in the prevention of stress fractures in both the elderly and elite female athletes. Given the incidence of disordered eating in this population and its effect on bone mineral density, this study provides more ammunition for convincing female athletes to forego energy and nutrient deficient diets in the quest for leanness.

Protocols will be difficult to develop unless testing and treatment become more standardized. Nielsen and Nightingale (47) recommend using a pharmaceutical iron preparation of 100 mg/d with a known high bioavailability for a period of 3 mo, with the conditions for testing standardized and reported. They feel that supplementation should be recommended for those athletes with serum ferritin <35 μg/L to reverse up-regulation of mineral absorption and prevent an increased absorption for other potentially toxic metals along with iron. The need for such high dosages should be assessed in future studies, especially when in cases of mild deficiency, 8–10 mg/d may be sufficient for repletion.

In the studies reviewed above, diagnosis of iron deficiency has been made with a range of serum ferritin levels (7 μg to 35 μg). Some of the more recent studies measured sTfR, others did not. Few controlled for diet or initial iron status. Some studies were conducted on trained individuals, others on individuals initiating an exercise program, and exercise protocols varied in intensity, duration, and frequency. As a result, clear recommendations are difficult to make.

PRACTICAL APPLICATIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Despite an upsurge in interest in physical activity, for most vitamins and minerals, current research is not conclusive enough to provide specific micronutrient recommendations to physically active women. It is clear that they need to get at least the RDA for micronutrients, and that many women fail to do so for several vitamins and minerals. Therefore, a primary practical implication is to assess the micronutrient intake of any women engaged in a physical activity program, or about to embark on a program, and make sure that her intake at least meets the RDAs.

With regard to iron and many other micronutrients, several articles in this supplement stress the importance of a woman being replete at the outset of her pregnancy. Although young female athletes may be focused on performance, and the idea of pregnancy may seem remote, the importance of educating them, their physicians, and their parents about starting pregnancy “with a full tank” of micronutrients, cannot be overemphasized. As to whether or not exercise increases micronutrient requirements, future studies should be designed to address the gaps that currently exist:

- variability of nutritional status of the participants at entry
- lack of control and standardization of subject’s dietary intake of the micronutrients of interest

REFERENCES


