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Back to the future\textsuperscript{1,2}

Dennis M Bier, Editor-in-Chief

In the fall of 1952, just shy of 55 years ago, the first issue of \textit{The Journal of Clinical Nutrition} appeared, preceding the founding of The American Society for Clinical Nutrition by 6 y. Renamed \textit{The American Journal of Clinical Nutrition} (AJCN) in 1954, the Journal has had a continuous, distinguished, and enviable record of scientific excellence under the guidance of only 8 Editors-in-Chief. I’ve had the pleasure to know 5 and be a friend of 3. Although it is a distinct honor to be the ninth Editor-in-Chief, it is likewise a humbling experience because of the accomplishments of those who have gone before me. Together, the former editors have made the AJCN the world’s most widely recognized, peer-reviewed clinical nutrition journal. Also, it is a truly daunting task to assume the editorship after the last Editor-in-Chief, Dr Charles Halsted, because he has accomplished nearly every goal that an editor of a scientific journal might strive for and has implemented essentially all of the improvements necessary to keep the AJCN both competitive among its peers in the current digital world and desirable to its diverse global readership. The success of his more than 11-year editorial tenure is amply demonstrated by the fact that the AJCN received 1270 original research manuscripts in 2006, 63% more than were submitted when Halsted assumed the editorship. Because of this high submission rate, the AJCN can be highly selective, accepting only 28% of the original research manuscripts received—a stringency that results in volumes of the highest scientific quality. The quality of the articles is supported objectively by the most recent AJCN impact factor of 5.853.

Where does one go from there? As attributed to many (1), “It is difficult to make predictions, especially about the future.” So a safer option is to look at the past. I did so to compose this editorial, and Halsted did so when the Journal turned 50 (2). Not surprisingly, we both realized that the initial editors “got it right,” in no small part because they recognized the value of nutrition research for preventing the diseases that are still the principal contributors to mortality worldwide and because they determined that the Journal would provide a forum for publication of research into the mechanisms responsible for their influence on disease prevention. Thus, the inaugural issue contains articles on the role of nutrition in hypertension, alcoholism, growth failure, vitamin A and amino acid deficiencies, low cholesterol diets, and “overnutrition and obesity”—topics that might easily appear in the AJCN today (2).

The opening article of the first issue in 1952, authored by Charles Glen King, was titled “Basic Research and its Application in the Field of Clinical Nutrition” (3). I find it impossible to improve on this title as the principal goal of the incoming editors. It is our firm belief that the single factor that determines the fate of a research journal is its scientific integrity. In this context, we define integrity as all the essential factors that, taken together, specify the validity of the material published therein. These factors include, but are not limited to, addressing issues of fundamental scientific merit in an unbiased manner by non-conflicted investigators and presenting the results from a balanced perspective after rigorous statistical scrutiny and critical peer review.

Although King (3) does not use the word \textit{translation} in his article, he would presumably be delighted to learn today that he advocated translational medicine before it was in vogue. Similarly, he would be happy to know that his 4 goals for nutrition scientists remain as pertinent today as they were when he wrote the inaugural article. Specifically, nutrition scientists “want to be able to identify and measure the concentration of all useful ingredients in human and animal foods. Second, they want to know how each nutrient functions in living organisms, and how these functions interlink, one with another. Third, they want to know the quantitative relations between nutrient intake and health on a life span basis. And fourth, they sense an obligation to assist in the educational activities that will make their work effective in the community at large.” Once again, this succinct statement needs no modification today. Perhaps most gratifying in the context of recent nutrition translation messages emphasizing foods and dietary patterns instead of nutrients, King develops the case that findings of good, translational nutrition research provide “strong support to the view that in the development of practical plans for good nutrition, one must look on the diet pattern as a whole and not be misled by overemphasis on the supply of individual nutrients at specific levels. The primary educational measures should be based on educating the public to consume liberal quantities of reliable protective foods” (3). Thus, I might add, \textit{Plus ça change…}, and argue that the ever rising and extending canopy of the AJCN evolutionary tree is supported by the depth of its initial roots.

The new editors have no uprooting plans. All the well-appreciated, established features of the AJCN will remain, including editorials, commentaries, and reviews. Likewise, the scope of the Journal will continue to cover the broadest range of clinical nutrition research themes. At the same time, the new

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editors appreciate that the sphere of nutritionally relevant sciences has surely expanded beyond the domains initially envisioned by the Journal’s founders, and we intend to make a serious effort to attract research articles of merit from all disciplines that have relevance to clinical nutrition. Acceptance of such articles will be limited only by scientific quality, not by topic. The new editors are also eager to expand the publication of systematic reviews of clinically relevant topics. In fact, a realistic goal is to become the principal source for such reviews in clinical nutrition and the repository of evidence-based classification of practice guidelines in this area. The editors are also discussing new features that we believe will be of interest to the readership, such as “Tools for Translation,” which will highlight new techniques or methods that should be applied more regularly to interdisciplinary nutrition research; “Tools for Interpretation,” which will analyze in detail the theoretical and practical strengths and weaknesses of the fundamental tools widely used in nutrition research; and “Boundaries of Our Knowledge,” which will be articles written by acknowledged leaders in specific fields of nutrition research describing the fundamental questions that must be answered or limitations that must be overcome to move the field forward. Finally, we are anxious to improve the Journal’s website by increasing the amount of information available and by allowing for the ability to download figures as PowerPoint slides, an option initiated by Halsted and already in the planning stage.

From a practical standpoint, the operations of the editorial office will be more widely distributed than in the past. The advances in electronic manuscript submission and processing that have taken place over the past decade make this possible. Thus, the new Associate Editors, all highly competent nutrition scientists in their own right, are geographically distributed: Naomi Fukagawa is on the faculty of the University of Vermont (Burlington, VT), Frank Sacks is a member of the Harvard University faculty (Boston, MA), Barbara Bowman is on the staff of the Centers for Disease Control and Prevention (Atlanta, GA), David Klurfeld is a scientist at the US Department of Agriculture/Agricultural Research Service (Beltsville, MD), Jonathan Gitlin is on the faculty of Washington University School of Medicine (St Louis, MO), and Steven Abrams is a member of the Baylor College of Medicine faculty (Houston, TX), as is the Editor-in-Chief. The Assistant Editor, D’Ann Finley, will remain at the University of California (Davis, CA). It is conceivable that more Associate Editors will be added as we gain experience with the specific areas of research expertise needed. Additionally, the new operational model for the Associate Editors is more closely aligned with that of The Journal of Nutrition, not only in view of their geographical distribution, but also because each Associate Editor will have direct responsibility for the individual articles assigned to him or her. The remaining members of the Editorial Board, who were appointed by Halsted, will complete their respective terms, and new members will be appointed as they retire.

The new editors are cognizant of the need for transparency with reference to potential conflicts of interest in the exercise of their responsibilities to the Journal, the authors, the referees, the American Society for Nutrition, and the reader. For this reason, we have developed a Conflict of Interest policy that also appears in this issue of the AJCN.

Finally, while looking forward to a bright future, I am not going to be driven by trying to second guess it. The best science is generally the most unexpected science. Rather, I expect to build on the foundations laid down by the Journal’s founders and will consider each day the first day of the future. This position is not particularly new, Horace (Odes, I-XI) stated such more than 2 millennia ago, “Dum loquimur, fugerit invida aetas: carpe diem, quam minimum credula postero,” “While we are talking, grudging time will have fled: seize the day, trusting as little as possible to the future.”

The author had no personal or financial conflict of interest relevant to the statements expressed in this article.

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Conflict of interest policy for Editors of The American Journal of Clinical Nutrition\textsuperscript{1,2}

Dennis M Bier, Editor-in-Chief; Steven A Abrams, Associate Editor; Barbara A Bowman, Associate Editor; Naomi K Fukagawa, Associate Editor; Jonathan D Gitlin, Associate Editor; David M Klurfeld, Associate Editor; and Frank M Sacks, Associate Editor

Integrity in the publication process requires impartiality at all levels of review. The American Journal of Clinical Nutrition (AJCN) adheres to the policy of the International Committee of Medical Journal Editors (ICMJE), Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Writing and Editing for Biomedical Publications (1). This policy details the ethical considerations relevant to ensuring impartiality. Consistent with this policy, the AJCN’s Information for Authors requires authors to disclose “any advisory board affiliations with and financial or personal interests in any organization sponsoring the research at the time the research was done.” Similarly consistent with the policy, the AJCN expects reviewers to recuse themselves from refereeing manuscripts in those circumstances in which a significant conflict of interest exists at either the financial or personal level. Furthermore, if a reviewer has knowledge of any relationship that might possibly constitute a conflict of interest when asked to evaluate a manuscript, it is the reviewer’s obligation to notify the editors, who will then decide whether to exclude the reviewer in that particular instance. Remarkably, there are few established, explicit conflict of interest policies for journal editors, although such an explicit policy was published recently by the Journal of Clinical Investigation (2) and is discussed further therein (3). The policy of the ICMJE (1) requires that editors “who make final decisions about manuscripts must have no personal, professional, or financial involvement in any of the issues they might judge.” Below, with acknowledgment to the editors of the Journal of Clinical Investigation for providing the framework (2), we, the new editors of the AJCN, provide our specific implementation of the ICMJE policy as it applies to our stewardship of the AJCN.

FINANCIAL CONFLICTS

AJCN Editors will declare, on the AJCN website (www.ajcn.org), all relationships from which they (and his or her spouse or dependent children) receive either assets or supplemental income of greater than $1000 per annum outside of compensation related to his or her full-time, permanent employment. In this context, a “relationship” is defined as 1) ownership of equity in any public or private company in the agriculture, food, nutrition, and pharmaceutical industries, but excluding holdings in mutual funds; 2) participation in any industry-related activity, agreement or arrangement that results in a financial payment of transfer of assets to the editor exceeding actual expenses for travel and participation; and 3) actual or in kind research support for the editor’s research activities. The initial website declaration will appear on 1 July 2007 and will include all potential conflicts that exist at that time. The website declaration will be updated yearly on July 1 to include the potential conflicts that have occurred in the intervening year. The initial conflict of interest declaration for new editors will be published on AJCN’s website when the editor assumes his or her duties and will be updated each year on July 1 thereafter.

OTHER CONFLICTS

Editors will recuse themselves from being responsible for manuscripts submitted by associates (former students, fellows, mentors, and collaborators) with whom they have worked over the previous 5 y and by faculty members at their own institutions. Manuscripts submitted to the AJCN by one of the editors will be handled by another editor. The AJCN’s electronic submission and review software allows the Associate Editor to deny the conflicted editor access to any information concerning manuscripts submitted by associates or by the individual editor. Additionally, the conflicted editor will be prohibited from participating in any discussion among the editors pertaining to such manuscripts.

In addition, the editors realize that it is not possible to define or anticipate every potential conflict. Thus, when new apparent conflicts arise, they will be evaluated individually by the Editor-in-Chief and by those Associate Editors who are not affected by the conflict. The affected editor agrees to abide by the decision of his or her colleagues in this instance. Finally, we should point out that conflict of interest policies are changing rapidly and that both

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the *AJCN* and the American Society for Nutrition will be reviewing and revising their overall conflict of interest policies this year. The *AJCN* Editors’ conflict of interest policy represents the first of several steps that are meant to keep nutrition research free of conflict of interest to the fullest extent possible.

None of the authors had any personal or financial conflicts of interest relevant to the conflict of interest policy expressed in this editorial.

**REFERENCES**

Adipose compartmentalization and insulin resistance among obese HIV-infected women: the role of intermuscular adipose tissue1,2

Tisha Joy and Steven K Grinspoon

Changes in adipose tissue volume and distribution have been linked to the development of insulin resistance and the metabolic syndrome in non-HIV-infected individuals. These changes may occur in conjunction with obesity or disorders of altered body fat distribution, often termed lipodystrophy. Indeed, the distribution of adipose tissue is an important determinant of metabolic risk, including insulin resistance. In this regard, both increases in visceral adipose tissue (VAT) and decreases in subcutaneous adipose tissue (SAT) are independently associated with metabolic abnormalities and increased insulin resistance in non-HIV-infected adults (1).

Among HIV-infected patients, metabolic abnormalities and changes in fat distribution are common. Approximately 20–50% of HIV-infected individuals will develop a change in adipose tissue distribution, dyslipidemia, or insulin resistance within 2 y of beginning antiretroviral therapy (2). Changes in adipose tissue in HIV-infected patients are typically manifested as peripheral fat loss, often in the lower extremities, with or without associated central fat gain. Both peripheral fat loss and visceral fat gain are independently associated with increased insulin resistance in this population (3, 4). Moreover, HIV-related adipose tissue changes are often associated with hypertriglyceridemia, low HDL concentrations, elevated free fatty acid (FFA) concentrations, and an elevated intramyocellular lipid (IMCL) content (2, 4, 5).

Early studies determining muscle attenuation from computed tomography (CT) in the HIV-infected population suggest that muscle adiposity (relative fat content of muscle in a specified area) is higher in HIV-infected individuals with increased truncal and reduced extremity fat than in HIV-infected patients without changes in fat distribution or in non-HIV-infected subjects. Moreover, it was shown that muscle attenuation, or degree of adiposity, was a strong independent predictor of hyperinsulinemia in the HIV-infected population with metabolic abnormalities (4). Subsequently, it was shown that muscle adiposity improved with exercise in association with improvements in insulin sensitivity in HIV-infected individuals (6). However, CT does not specifically quantitate the IML content of muscle adipose tissue, whereas magnetic resonance spectroscopy does.

Among HIV-infected patients, Gan et al (7) found that increased visceral fat was strongly associated with IMCL concentrations, as assessed by magnetic resonance spectroscopy. Increased IMCL content was a strong predictor of insulin-stimulated glucose disposal during a hyperinsulinemic euglycemic clamp. A model for the pathogenesis of insulin resistance in HIV-infected individuals was proposed by Balasubramanyam et al (8). In this model, dysregulation of fatty acid metabolism in peripheral fat depots is hypothesized to contribute to increased lipolysis and increased circulating FFA concentrations. The increased FFA transport into skeletal muscle is thought to result in increased intramyocellular concentrations of fatty acyl coenzyme A, increased IMCL deposition, and suppression of insulin-mediated glucose transport into skeletal muscle, which thereby induces insulin resistance (8). Data in support of this hypothesis, recently published by Hadigan et al (9), indicate that treatment with acipimox (a nicotinic acid analog not currently approved by the Food and Drug Administration in the United States) to inhibit lipolysis improved insulin sensitivity while decreasing the IMCL content.

Recently, the accumulation of intermuscular adipose tissue (IMAT) was recognized as an important determinant of insulin resistance. IMAT refers to the adipose tissue between muscle fibers, whereas IMCL refers to the adipose tissue within muscle. Measurement of IMAT was initially based on the CT measurement of decreased attenuation. Subsequently, IMAT was assessed more directly with magnetic resonance imaging (MRI). Goodpaster et al (10) found that intermuscular and intramuscular fat are independently and positively associated with insulin resistance in obese nondiabetic and diabetic non-HIV-infected individuals. Recently, Albu et al (11) showed that whole-body IMAT, assessed by using MRI, was a predictor of insulin resistance in non-HIV-infected women, independent of race, weight, height, and total skeletal muscle volume. These data highlight the importance of intermuscular adipose tissue accumulation as a critical factor regulating glucose trafficking.

What is the influence of IMAT on insulin sensitivity among HIV-infected patients? In this issue of the Journal, Albu et al (12) present the results of a cross-sectional study that compared the relation of adipose distribution with insulin resistance in 17 obese HIV-infected women with that in 32 obese but otherwise healthy HIV-negative controls. Whole-body MRI was used to determine SAT, VAT, and whole-body IMAT. Insulin sensitivity was assessed by intravenous glucose tolerance testing. HIV-infected women had relatively less SAT but more VAT and IMAT than...
did HIV-negative women. Increased whole-body IMAT and reduced leg SAT were independent correlates of insulin resistance in HIV-infected women in this cross-sectional study.

This study by Albu et al provides important new information regarding the assessment of IMAT in obese HIV-infected women and the relation of IMAT to insulin resistance in this population. Taken together with prior data in non-HIV-infected women, the studies of Albu et al suggest that IMAT appears to have a strong independent effect on insulin resistance in women in general and greater effects in HIV-infected patients, in whom IMAT accumulation is excessive. In addition, the inverse relation between leg SAT and insulin resistance shown in HIV-infected women supports the model of insulin resistance proposed by Balasubramanyam et al (8) Specifically, decreased storage capacity of fat in leg SAT could lead to the “spillover” of fatty acids into plasma and ultimately into skeletal muscle, which would contribute to insulin resistance. Whether this “spillover” is responsible for the increase in IMAT and for the significant association between IMAT and insulin resistance remains to be elucidated.

The current study by Albu et al has some important limitations. It was conducted in obese HIV-infected women who had been recruited for a study on weight loss, which limited the generalizability of the findings to this specific subpopulation of HIV-infected patients. Furthermore, the study provided little information on the effect of IMAT on other metabolic variables, including lipid concentrations, and no information on physical activity and dietary intake. Specifically, it is not clear whether physical activity or dietary intake differed between the 2 groups and, if so, whether such differences account for the variations in adipose deposition and insulin resistance. Further studies are needed to investigate the relation between reduced SAT, increased IMAT, and dyslipidemia. Similarly, longitudinal studies of the effect of specific antiretroviral medications on IMAT are needed. Nonetheless, the current study provides new information regarding the importance of intermuscular adipose tissue accumulation in the HIV population receiving antiretroviral therapy.

Neither author had a conflict of interest pertaining to this article.

REFERENCES

Alternate-day fasting and chronic disease prevention: a review of human and animal trials1–3

Krista A Varady and Marc K Hellerstein

ABSTRACT
Calorie restriction (CR) and alternate-day fasting (ADF) represent 2 different forms of dietary restriction. Although the effects of CR on chronic disease prevention were reviewed previously, the effects of ADF on chronic disease risk have yet to be summarized. Accordingly, we review here animal and human evidence concerning ADF and the risk of certain chronic diseases, such as type 2 diabetes, cardiovascular disease, and cancer. We also compare the magnitude of risk reduction resulting from ADF with that resulting from CR. In terms of diabetes risk, animal studies of ADF find lower diabetes incidence and lower fasting glucose and insulin concentrations, effects that are comparable to those of CR. Human trials to date have reported greater insulin-mediated glucose uptake but no effect on fasting glucose or insulin concentrations. In terms of cardiovascular disease risk, animal ADF data show lower total cholesterol and triacylglycerol concentrations, a lower heart rate, improved cardiac response to myocardial infarction, and lower blood pressure. The limited human evidence suggests higher HDL-cholesterol concentrations and lower triacylglycerol concentrations but no effect on blood pressure. In terms of cancer risk, there is no human evidence to date, yet animal studies found decreases in lymphoma incidence, longer survival after tumor inoculation, and lower rates of proliferation of several cell types. The findings in animals suggest that ADF may effectively modulate several risk factors, thereby preventing chronic disease, and that ADF may modulate disease risk to an extent similar to that of CR. More research is required to establish definitively the consequences of ADF.

KEY WORDS Alternate-day fasting, calorie restriction, type 2 diabetes, cardiovascular disease, cancer, animal models, humans

INTRODUCTION
Calorie restriction (CR), defined as a reduction in energy intake without malnutrition, has been shown to increase life span, improve numerous functional indexes, and reduce metabolic risk factors for chronic disease in several mammalian species (1, 2). CR regimens have consisted of reducing food intake to 60–85% of daily energy needs. As an alternative to traditional CR, another dietary regimen, termed alternate-day fasting (ADF), has also been tested. ADF regimens generally involve a “feast day” on which food is consumed ad libitum that alternates with a “fast day” on which food is withheld or reduced. The feast and fast periods are typically 24 h each, but they may vary. A key point about the ADF approach is that overall calorie intake need not be limited; instead, the frequency of food consumption is altered (3).

The purpose of this review is to summarize the relatively sparse but highly suggestive literature on ADF regimens. Although the effects of CR on chronic disease prevention were discussed in reviews conducted in the past few years (4–6), the ability of ADF to alter chronic disease risk has not yet been summarized. In particular, the key question—whether ADF has effects on risk modulation comparable to those of CR—remains uncertain. Accordingly, our objective was to review the evidence from both animal and human trials concerning ADF and the risk of chronic diseases, such as type 2 diabetes mellitus, cardiovascular disease (CVD), and cancer. In addition, when possible, the magnitude of risk reduction due to ADF will be compared with that due to CR.

BENEFITS OF CALORIE RESTRICTION REGIMENS
A large body of evidence for the physiologic benefits and life-extending properties of CR now exists. Restricting daily energy intake by 15–40% has been shown in both animals and humans to improve glucose tolerance and insulin action, which indicates an enhancement in insulin sensitivity (7, 8); to reduce blood pressure and the heart rate, which is consistent with benefits for cardiovascular health (9–11); and to reduce oxidative damage to lipids, protein, and DNA, which implies a protective effect against oxidative stress (12–15). Many other effects of CR have been documented, including increased average and maximal life span (12), reduced incidence of spontaneous and induced cancers (13), resistance of neurons to degeneration (14), lower rates of kidney disease (15), and prolongation of reproductive function (16).

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Although the precise mechanisms responsible for such effects are still not clear, several general hypotheses have been proposed—most prominent are the stress resistance hypothesis, the oxidative stress hypothesis, and the induction of a scarcity program hypothesis (3, 17–19). The first hypothesis suggests that, after prolonged dietary restriction, increased resistance to different types of stressors occurs, which permits the cells of many tissues to resist injury induced by genotoxic, metabolic, or oxidative insults (20–22). The second hypothesis proposes more specifically that fewer free radicals are produced in the mitochondria of cells, because dietary restriction generally limits energy utilization, which results in less cellular oxidative damage (3). The third hypothesis proposes that CR induces intrinsic cellular and organismal programs for adaptation to scarcity, which result in the slowing of metabolic processes such as cell proliferation that contribute to senescence; this hypothesis has been strengthened by findings in yeast (19). The effects of ADF on these proposed mechanisms have not been explored as extensively as have the effects of CR, but some evidence has been generated, and that will be reviewed here.

EFFECT OF ALTERNATE-DAY FASTING ON TYPE 2 DIABETES RISK

Animal studies

To date, 12 studies using animal models have examined the effect of ADF on chronic disease risk (23–34; Table 1). Approximately half of these studies examined variables related to diabetes, such as fasting glucose and insulin concentrations, fat oxidation, degree of insulitis, and occurrence of type 2 diabetes. Fasting glucose concentrations have generally been reported to decrease in response to ADF in animal models. Three studies found reduced circulating glucose concentrations after a 20–24-wk intervention (27, 30), whereas one study reported no effect on glucose concentrations after a 16-wk treatment (24). In the trials that measured insulin concentrations, consistent reductions were noted after ADF regimens that lasted 20 (27) and 24 (28) wk. It is interesting that, in the study of Anson et al (27), both glucose and insulin concentrations decreased to a similar extent in the ADF and the 40% CR groups. Increases in fat oxidation in liver and muscle have also been observed after relatively short periods (8 wk) of ADF (33).

Because impaired fat oxidation may contribute to ectopic accumulation of intracellular lipid and the development of insulin resistance (35), these increases in fat oxidation may increase insulin sensitivity. Also noted by Anson et al was a doubling of the plasma concentrations of β-hydroxybutyrate in the ADF group but no change in the control group. In contrast, concentrations of this metabolite decreased in the 40% CR group but not in the control group (27). These results suggest that high rates of fatty acid oxidation leading to ketogenesis occurred with ADF but not with 40% CR. Moreover, reduced occurrence of insulin-dependent diabetes in response to ADF has been reported by Pedersen et al (30). These authors found that 77% of the BB rats fed ad libitum control diets developed diabetes, whereas only 52% of the animals fasted for 24 h on alternate days became diabetic. The degree of insulitis, however, was not affected, which suggested that the mechanism most likely did not involve modulation of this inflammatory variable (30).

Human trials

Risk factors for type 2 diabetes were measured in each of the 3 published human studies of ADF (36–38; Table 2). Evidence from these trials suggests that ADF does not alter fasting concentrations of glucose but may beneficially modulate other indexes of diabetes risk, such as insulin sensitivity. Specifically, Halberg et al (38) observed that, when normal-weight persons fasted for 20-h periods (fast day) and then ate their habitual diet ad libitum on alternate days (feast day), the insulin-mediated glucose uptake increased after 2 wk of intervention, as measured by using the euglycemic-hyperinsulinemic clamp technique. These results are supported by a study conducted by Heilbronn et al (36), which found that, after 3 wk of ADF, insulin response to a test meal was reduced, which implied improved insulin sensitivity. It is interesting that this effect on insulin sensitivity occurred only in male subjects (36). Another diabetes risk factor that has shown a sex-specific effect is glucose tolerance. After 3 wk of ADF, women but not men had an increase in the area under the glucose curve (36). This unfavorable effect on glucose tolerance in women, accompanied by an apparent lack of an effect on insulin sensitivity, suggests that short-term ADF may be more beneficial in men than in women in reducing type 2 diabetes risk. However, because minimal data and no longer-term studies are available to support this important hypothesis, more studies are needed. The effect of ADF regimens on insulin concentrations appears equivocal (37, 38). Specifically, Halberg et al (38) found that 2 wk of ADF had no effect on fasting insulin concentrations, whereas Heilbronn et al (37) found that 3 wk of this intervention decreased insulin concentrations, but only after a 32-h fast. Further research examining the time course of ADF effects on such diabetes-related variables could help clarify this matter. Also examined was the responsiveness of skeletal muscle and adipose tissue to ADF (38). A 2-wk ADF regimen had no effect on intramuscular triacylglycerol (IMTG) concentrations in normal-weight men (38). In adipose tissue, an inhibitory effect of insulin on adipose tissue lipolysis was observed after 2 wk of intervention (38). Because increased concentrations of free fatty acids have been implicated in the pathogenesis of type 2 diabetes (39), this decrease in lipolysis and circulating concentrations of free fatty acids may represent an indirect protective effect of ADF on diabetes risk.

EFFECT OF ALTERNATE-DAY FASTING ON CARDIOVASCULAR DISEASE RISK

Animal studies

As a means of assessing cardiovascular response to ADF, trials in this area have examined heart rate, blood pressure, circulating lipids, and ischemic injury. In a recent study by Mager et al (24), reductions in heart rate were observed in Sprague-Dawley rats after 16 wk of ADF. Similar effects on heart rate were also found by Wan et al (28) after 24 wk of ADF. In both of these trials, treatment-induced decreases in systolic and diastolic blood pressure were noted after 4 wk, and the lower blood pressures persisted throughout the course of the studies. Moreover, the magnitude of the effect on heart rate and blood pressure was similar in the ADF group and a 40% CR group, which suggests that ADF may be as beneficial as CR in modulating these variables (24).
Animal trials examining the effect of alternate-day fasting (ADF) on chronic disease risk factors

Table 1: Effect of ADF on risk factors

<table>
<thead>
<tr>
<th>Reference</th>
<th>Animal model</th>
<th>Trial length</th>
<th>Intervention groups</th>
<th>Weight change</th>
<th>DM</th>
<th>CVD</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsieh et al, 2006 (23)</td>
<td>n = 36 C57BL6 mice Age 2 mo</td>
<td>12 wk</td>
<td>1) ADF (n = 6) 2) 33% CR (n = 6) 3) 33% CR (n = 6)</td>
<td>↓↑</td>
<td>Not measured</td>
<td>Not measured</td>
<td>Mammary epithelial cell proliferation: ↓ Spleenic T cell proliferation: ↓ Skin epithelial cell (keratinocyte) proliferation: ↓</td>
</tr>
<tr>
<td>Mager et al, 2006 (24)</td>
<td>n = 12 SD rats Age 2 mo</td>
<td>16 wk</td>
<td>1) ADF (n = 6) 2) 40% CR (n = 6)</td>
<td>None</td>
<td>Fasting glucose: none</td>
<td>Heart rate: ↓ Blood pressure: ↓</td>
<td>Not measured</td>
</tr>
<tr>
<td>Ahmet et al, 2005 (25)</td>
<td>n = 60 SD rats Age 2 mo</td>
<td>12 wk</td>
<td>1) ADF (n = 30) 2) Control (n = 30)</td>
<td>↓↑</td>
<td>Not measured</td>
<td>MI induced (wk 12) 24 h after MI: MI size: 50% smaller Apoptotic myocytes number 75% less Inflammatory response after MI: ↓↓</td>
<td></td>
</tr>
<tr>
<td>Descamps et al, 2005 (26)</td>
<td>n = 30 OFl mice Age 6 mo</td>
<td>16 wk</td>
<td>1) ADF (n = 15) 2) Control (n = 15)</td>
<td>None</td>
<td>Not measured</td>
<td>Not measured</td>
<td>Incidence of lymphoma: 0% (incidence in control: 33%) Spleen ROS generation: ↑↑ Spleen SOD activity: ↑↑↑ Spleen GSH/GSSG ratio: ↑↑↑ MDA concentrations: none</td>
</tr>
<tr>
<td>Anson et al, 2003 (27)</td>
<td>n = 24 C57BL6 mice Age 2 mo</td>
<td>20 wk</td>
<td>1) ADF (n = 8) 2) 40% CR (n = 8) 3) Control (n = 8)</td>
<td>None</td>
<td>Fasting glucose: ↑ Fasting insulin: ↑ β-Hydroxybutyrate: ↑↑ IGF-1 concentrations: ↑↑ IGF-1 concentrations: ↑↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wan et al, 2003 (28)</td>
<td>n = 24 SD rats Age 3 mo</td>
<td>24 wk</td>
<td>1) ADF (n = 8) 2) 2DG suppl (n = 8) 3) Control (n = 8)</td>
<td>↓↑</td>
<td>Fasting glucose: ↑ Fasting insulin: ↑↑ IGF-1 concentrations: ↑↑ IGF-1 concentrations: ↑↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rocha et al, 2002 (29)</td>
<td>n = 24 Wistar rats Age 2 mo</td>
<td>48 wk</td>
<td>1) ADF (n = 12) 2) Control (n = 12)</td>
<td>None</td>
<td>Not measured</td>
<td>Not measured</td>
<td>After DEN injection to initiate liver carcinogenesis: Development of hepatic preneoplastic lesions inhibited Decreased size and number of hepatic nodules</td>
</tr>
<tr>
<td>Pedersen et al, 1999 (30)</td>
<td>n = 161 BB rats Age 3 mo</td>
<td>20 wk</td>
<td>1) ADF (n = 44) 2) Fast for 24 h 2x/wk (n = 40) 3) Control (n = 77)</td>
<td>↓↑</td>
<td>DM incidence: 52% (DM incidence in control group: 79%) Degree of insulitis: none Fasting glucose: ↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Krizova and Simek, 1996 (32)</td>
<td>n = 30 C17/B1-10 mice Age 2 mo</td>
<td>8 wk</td>
<td>1) ADF–regular diet on feed day (n = 10) 2) ADF–40% fat diet on feed day (n = 10) 3) Control (n = 10)</td>
<td>↑↑↑↑↑↑ (both groups)</td>
<td>Not measured</td>
<td>Total cholesterol: ↑ Triacylglycerols: ↑↑ (both groups)</td>
<td>Not measured</td>
</tr>
<tr>
<td>Krizova and Simek, 1996 (33)</td>
<td>n = 30 C17/B1-10 mice Age 2 mo</td>
<td>8 wk</td>
<td>1) ADF–regular diet on feed day (n = 10) 2) ADF–high-fat diet on feed day (n = 10) 3) Control (n = 10)</td>
<td>↑↑↑↑↑↑ (both groups)</td>
<td>Liver fat oxidation: ↑↑ Muscle fat oxidation: ↑↑ (both groups)</td>
<td>Not measured</td>
<td>Not measured</td>
</tr>
<tr>
<td>Goodrick et al, 1990 (31)</td>
<td>n = 120 A/J or C57BL/6J mice Age 1 mo</td>
<td>Until death</td>
<td>1) ADF at age 2 mo (n = 30) 2) ADF–starting at age 6 mo (n = 30) 3) ADF–starting at age 10 mo (n = 30) 4) Control (n = 30)</td>
<td>↓↑ (C57 groups)</td>
<td>Not measured</td>
<td>Not measured</td>
<td>C57BL/6J mice: ↑↑↑↑↑↑ (all ADF groups) A/J mice: Life span: ↑↑↑↑↑↑ (2 mo group) Life span: none (6 mo group) Life span: ↓↓↓↓ (10 mo group)</td>
</tr>
<tr>
<td>Seigel et al, 1988 (34)</td>
<td>n = 48 Fisher rats Age 3 mo</td>
<td>2 wk</td>
<td>1) ADF (n = 24) 2) Control (n = 24)</td>
<td>None</td>
<td>Not measured</td>
<td>Not measured</td>
<td>After tumor inoculation (wk 1): 12/24 ADF rats survived for 10 d after inoculation (compared to 3/24 controls)</td>
</tr>
</tbody>
</table>

In addition, Krizova and Simek (32) observed decreases in circulating lipid concentrations, and, after 8 wk of ADF, both total cholesterol and triacylglycerol concentrations decreased in adult mice. Moreover, the cardiac myocyte response to myocardial infarction (MI) induction has been studied by Ahmet et al (25). MI was induced by coronary artery ligation after 12 wk of ADF or control ad libitum diet in Sprague-Dawley rats. At 24 h after MI induction, the number of apoptotic myocytes in the affected area was one-fourth that in the ad libitum–fed controls, and the size of the MI in the ADF group was half that in the ad libitum–fed controls. Also noted was a distinct reduction in neutrophil infiltration, which suggested a decrease in inflammatory response (25).

Notes:
1. 2DG suppl, supplementation with 2-deoxy-D-glucose; BB, Bio-breeding; BW, body weight; CR, calorie-restricted; CVD, cardiovascular disease; DEN, diethylnitrosamine; DM, diabetes mellitus; GSH/GSSG, glutathione/glutathione disulfide; IGF-1, insulin-like growth factor-1; MDA, malondialdehyde; ROS, reactive oxygen species; SD, Sprague-Dawley; SOD, superoxide dismutase.
2. ADF group, fed ad libitum for 24 h and then fasted for 24 h; control group, fed ad libitum daily.
3. Posttreatment values of ADF group significantly different from posttreatment values of control group, P < 0.05.
4. Posttreatment values significantly different from baseline values within the ADF group, P < 0.05.
Human trials

Heilbron et al (37) examined the effect of ADF on CVD risk. When human subjects fasted on alternate days for a short period (3 wk), circulating concentrations of HDL cholesterol increased, whereas triacylglycerol concentrations decreased (37). It is possible that these effects resulted from the decreases in body weight (2.5%) and fat mass (4.0%) observed in these subjects, who were unable to consume sufficient calories on the fast day to maintain an isocaloric state (37). It is interesting that the shifts in lipid concentrations were shown to be sex specific: ie, only the women had an increase in HDL-cholesterol concentrations, and only the men had a decrease in triacylglycerol concentrations (37). There is no clear explanation for these sex-based differences. The effect of ADF on blood pressure was also examined in this study. After 3 wk of intervention, neither systolic nor diastolic blood pressure changed in either the male or female subjects (37). This study included only normotensive persons, however. It will be of interest in future studies to examine the effect of this dietary regimen on blood pressure in other patient groups, such as those who are hypertensive, overweight, obese, and hyperlipidemic.

EFFECT OF ALTERNATE-DAY FASTING ON CANCER RISK: ANIMAL TRIALS

The protective effect of ADF on cancer survival was first described by Seigel et al (34). In this study, 3- to 4-mo-old rats were administered an ADF regimen beginning 1 wk before inoculation with MAT 13762 acites tumor cells (34). Twelve of the 24 rats (50%) in the diet-restricted group survived 10 d after tumor inoculation, in comparison with only 3 of 24 animals (12.5%) in the control group, which had been fed ad libitum (34). The response to ADF of certain biomarkers of cancer risk, ie, insulin-like growth factor-1 (IGF-1), has also been investigated (27, 28), but the results have been inconsistent. Whereas Anson et al (27) reported increases in IGF-1 in response to 20 wk of ADF, Wan et al (28) reported a clear decrease after 24 wk of treatment. IGF-1 is a potent promoter of cell proliferation and has been shown to decrease in response to CR (40). The animals in the study of Anson et al (27) did not lose any weight, whereas animals in the study of Wan et al (28) did lose weight; the lack of overall negative energy balance may explain the different IGF-1 responses in those studies.

More recently, the protective effect of the ADF restriction protocol on age-associated lymphoma and hepatocarcinogenesis was examined in mice (26). After 16 wk, the incidence of lymphoma in OF1 mice administered an ADF regimen was 0% and that in the control group was 33%. Because the ADF group mice consumed roughly the same total amount of food as the control mice, the efficacy of ADF was independent of total calorie intake (26). Also noted in that study was a significant, treatment-induced increase in spleen mitochondrial superoxide dismutase (SOD) activity, which was associated with reduced mitochondrial generation of reactive oxygen species (ROS). The effect of ADF on hepatocarcinogenesis has also been examined (29) after 4 wk of ad libitum feeding. In that study, Wistar rats were injected with diethylnitrosamine to initiate liver carcinogenesis and then fed on alternate days for 48 wk. When compared with ad libitum feeding, ADF inhibited the development of preneoplastic lesions and also decreased the number and size of liver nodules (29). These findings strongly support the hypothesis that long-term ADF may exert an antipromotional effect on experimental carcinogenesis, as has been shown in many studies of CR. Moreover, strong physiologic evidence in favor of the antipromotional effects of ADF was recently reported by Hsieh et al (23). After 12 wk of treatment, reduced rates of proliferation of several cell types, including mammary epithelial cells, skin epithelial cells (keratinocytes), and splenic T-cells, was observed (23). These changes induced by ADF were similar to, though not quite as potent as, those seen in the CR groups.

ADF regimens have also been shown to increase mean and maximal life span in certain strains of mice (31). When ADF regimens were initiated in C57BL/6j mice at ages 2, 6, and 10 mo, body weight decreased and maximal life span was extended (31). It is interesting that, when the same ADF protocol was administered to A/J mice, body weight was not affected, and life span was increased only in the group that began ADF at age 2 mo (31). These findings suggest that ADF-induced changes in body weight and maximal life span may be strongly influenced by genotype and the age at which ADF is initiated.
EFFECT OF ALTERNATE-DAY FASTING ON OTHER METABOLIC VARIABLES: HUMAN TRIALS

The effect of short-term ADF regimens on other metabolic variables, such as body temperature, resting metabolic rate (RMR), and various hormone and cytokine concentrations, has also been examined in human subjects (36, 37). In the study by Heilbronn et al (37), neither body temperature nor RMR was affected by 3 wk of treatment. On the other hand, overall fat oxidation was shown to increase by an average of 15 g/d over the course of the trial, according to indirect calorimetry. The authors also observed a positive correlation between fat oxidation and weight loss, which suggested that those subjects with a greater ability to oxidize fat may have lost more weight (37). Thus, whether the weight loss noted is a result of ADF may depend on a person’s ability to oxidize fat. Heilbronn et al (36) also examined treatment-induced changes in the expression of certain skeletal muscle genes involved in fat oxidation, including β-hydroxyacyl CoA dehydrogenase, fatty acid translocase, pyruvate dehydrogenase kinase 4, carnitine palmitoyltransferase 1, and uncoupling protein 3, as well as the expression of genes implicated in mitochondrial biogenesis, including peroxisome-proliferator-activated receptor-γ co-activator 1, nuclear respiratory factor 1, and cytochrome C. They reported that a 3-wk treatment had no effect on the expression of any of these genes. The response of circulating concentrations of certain adipokines—ie, adiponectin, leptin, interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α)—has also been examined (38). Concentrations of leptin, IL-6, and TNF-α did not change in response to ADF; in contrast, concentrations of adiponectin increased by 37% (38). Cnop et al (41) and Higashiura et al (42) both reported that circulating concentrations of adiponectin are positively correlated with insulin sensitivity, which suggests a possible role of this adipokine in the insulin-sensitizing effect noted in the present trial (38).

SUMMARY OF FINDINGS: ALTERNATE-DAY FASTING IN HUMAN AND ANIMAL TRIALS

Some discrepancies between human and animal ADF data are evident. With regard to the effect of ADF on the risk of type 2 diabetes mellitus, the results to date from human trials have been inconsistent, whereas the animal evidence suggests favorable alterations. Fasting glucose concentrations in rodents, for example, consistently were lower after 20 wk of treatment (27, 28, 30), whereas, in humans, no effect was seen after 2–3 wk of ADF (37, 38). It may be that longer intervention periods are required to alter glucose concentrations in human subjects. In the case of fasting insulin concentrations, equivocal findings were noted in humans (37, 38), whereas consistent decreases have been seen in animals (27, 28). Moreover, animal data indicate that ADF is just as efficacious in decreasing fasting glucose and insulin concentrations as is daily CR (27). Although neither glucose nor insulin concentrations were affected in the brief human trials carried out to date, it is interesting that findings such as increased insulin-mediated glucose uptake and reduced adipose tissue lipolysis have been reported (38). In animal models, fatty acid oxidation in liver and muscle is increased with ADF (33); this may indicate that ectopic accumulation of intracellular lipid could be decreased, which in turn may lead to improved insulin sensitivity (43). Nevertheless, it should be noted that, in obese human subjects, CR does not affect intramyocellular lipid content but does result in decreased lipid accumulation in liver (44). Complementary to this evidence, the incidence of type 2 diabetes risk was lower in rodents fed on alternate days than in ad libitum–fed controls (30). In sum, the favorable effects noted in animal studies suggest that prolonged ADF is a beneficial means of lowering type 2 diabetes risk. Results from human studies, however, are less clear. It seems reasonable to expect that ADF will improve insulin sensitivity in humans, but the conflicting findings make it difficult to be certain about this. Longer intervention trials (ie, ≥20 wk) in human subjects may help to clarify this issue.

Although overall beneficial modulations in risk factors for vascular disease have been found, with respect to blood pressure, the evidence from animal studies has shown a consistent decrease in both systolic and diastolic readings (24, 28), whereas data from human trials have shown no effect on either variable (37). One possible explanation for this inconsistency may be the differences in the duration of intervention. Because an effect on blood pressure readings was identified only after a 4-wk treatment in animals (24, 28), treatment for 3 wk in the human study may not have been long enough. Alternatively, weight loss, ie, negative energy balance, may be required for a blood pressure effect in humans. In the case of circulating lipid concentrations, beneficial modulations have been noted in both human and animal studies (32, 37). Specifically, human data show treatment-induced increases in HDL-cholesterol concentrations and reductions in triacylglycerol concentrations (37), and results in rodents show decreases in total cholesterol and triacylglycerol concentrations (32). Also shown, although only in rodent models, were improvements in cardiac response to MI induction (25) and decreases in heart rate (24, 28). In addition, the decreases in heart rate and blood pressure induced by ADF were similar to those induced by CR (24). Taken together, these improvements suggest that ADF may help reduce the risk of CVD.

To date, the direct effect of ADF on cancer has been tested only in animal models. Most of those trials suggest a pronounced beneficial effect on cancer risk factors, including substantially decreases in lymphoma incidence (26), increases in spleen SOD activity accompanied by reductions in ROS generation (26), inhibition of hepatic preneoplastic lesion development (29), and a greater survival rate after tumor inoculation (34). The physiologic evidence of clear reductions in proliferation rates of several cell types—including mammary epithelial cells, keratinocytes, and splenic T-cells—induced by ADF regimens also supports the antipromotional actions of this intervention (23). The antiproliferative mechanism remains unknown, however, because the effects of ADF on IGF-1 concentrations have been inconsistent (27, 28). Nevertheless, most of these studies have reported a protective effect, so it is reasonable to propose that ADF will prove to be an effective means of decreasing cancer risk. Studies in human subjects are still required to answer this important question.

An interesting but unresolved issue is the effect of ADF on body weight. Body weight has been shown to be highly variable in response to ADF in both human and animal models. In some animal models, when ADF regimens have been applied in the short term, no effect on body weight has been noted after 2 wk (34), whereas gains in weight were noted in other trials after 8 wk (32, 33). It is possible that the animals in the 8-wk trial may have overcompensated for the lack of food on the fast day by eating more than twice their average daily intake on the feast day. In other studies, when ADF regimens were administered for 12 wk,
body weight was found to decrease (23, 25), but, when ADF regimens were administered for 16 wk, no effect on body weight was observed (24, 26). Trials examining the effects of long-term (>20 wk) ADF in animals (28–31) have fairly consistently found decreases in body weight, although the study by Anson et al (27) did not. Such findings suggest that the animals were unable to consume twice their daily food intake on the feast day for longer periods, which resulted in a loss of body weight. There clearly is variability in the capacity of animals, even within the same strain of mouse, to compensate for a fast day on the feast day (27). A variety of factors, such as housing conditions, palatability, or energy density of diet, and genetics can be hypothesized as influencing compensation. Understanding the factors controlling compensation is an important area for future research.

The effect of ADF on body weight in humans is difficult to infer because of the very short trial durations and the small number of studies published to date. As was seen in animal trials, 2 wk of ADF had no effect on body weight in normal-weight human subjects (38). Nevertheless, when the intervention period in humans was extended to 3 wk, a decrease in body weight (∼2.5 kg) was noted (36, 37). This decrease in body weight may have resulted from an inability to consume an adequate amount of food on the feast day to sustain body weight. Similar findings were noted in animals, but only after much longer trial durations (>20 wk). An important study design issue is whether weight loss should be prevented in human ADF studies by “forced” maintenance of calorie intake. Forcing maintenance of intake may produce harmful effects over the long term, so this approach cannot currently be recommended. It is also possible that weight loss in humans following an ADF regimen will prove to be minor or transient. In contrast, striving for full compensation of calorie intake (ie, no weight loss) in studies lasting only weeks or a few months is unlikely to have significant adverse health consequences. The absence of weight loss would allow useful comparisons and distinctions between ADF and CR. These issues will require consideration by investigators who conduct future human trials with ADF.

CONCLUSIONS

Findings to date from both human and animal experiments indicate that ADF may effectively decrease the risk of CVD, whereas results from animal studies suggest a protective effect on cancer risk. In terms of diabetes prevention, animal data suggest a beneficial effect, but human data have been equivocal. However, it is important to note that the human studies examined in this review are limited; they all lacked control groups and used short trial lengths. Future studies with longer trials and including control groups are needed to answer these important questions. The effect of ADF regimens in insulin-resistant or diabetic populations also should be determined, because they could help to clarify the role of ADF as a treatment for preexisting diabetes rather than as a protection against diabetes.

Moreover, human ADF trials in modestly overweight persons, who are at greater risk of chronic disease, are warranted. In this context, it is important to note that the control animals in both the CR and ADF studies are likely to have been obese, because they were fed ad libitum.

ADF regimens also may be as efficacious as daily CR in improving certain indexes of risk of type 2 diabetes and CVD, although the number of studies directly comparing the 2 regimens is small. Further analysis of the mechanisms responsible for beneficial effects of ADF is clearly warranted, particularly if these effects occur in the absence of negative energy balance. Novel mediators and therapeutic strategies may thereby be uncovered. Finally, it seems intuitively likely that persons will find it easier to fast or reduce intake on alternate days than to reduce their intake every day. For this reason, ADF regimens may allow better compliance than would CR regimens and may represent an attractive area for investigation.

It will also be important to understand whether the mechanisms by which ADF protects against chronic disease risk are similar to those of CR. Indirect evidence suggests that the 2 regimens may share mechanisms. For instance, the study of Descamps et al (26) reported increases in spleen mitochondrial SOD activity accompanied by decreases in mitochondrial generation of ROS as a result of ADF. Such findings suggest that ADF may act by increasing resistance to oxidative insult, which is a key feature of the stress resistance hypothesis.

In summary, this still nascent literature suggests that ADF may effectively modulate metabolic and functional risk factors, thereby preventing or delaying the future occurrence of common chronic diseases, at least in animal models. The effect of ADF on chronic disease risk in normal-weight human subjects remains unclear, however, as do the mechanisms of action. Much work remains to be done to understand this dietary strategy fully.

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REFERENCES


Role of S-adenosylmethionine, folate, and betaine in the treatment of alcoholic liver disease: summary of a symposium¹–⁴

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ABSTRACT
This report is a summary of a symposium on the role of S-adenosylmethionine (SAM), betaine, and folate in the treatment of alcoholic liver disease (ALD), which was organized by the National Institute on Alcohol Abuse and Alcoholism in collaboration with the Office of Dietary Supplements and the National Center for Complementary and Alternative Medicine of the National Institutes of Health (Bethesda, MD) and held on 3 October 2005. SAM supplementation may attenuate ALD by decreasing oxidative stress through the up-regulation of glutathione synthesis, reducing inflammation via the down-regulation of tumor necrosis factor-α and the up-regulation of interleukin-10 synthesis, increasing the ratio of SAM to S-adenosylhomocysteine (SAH), and inhibiting the apoptosis of normal hepatocytes and stimulating the apoptosis of liver cancer cells. Folate deficiency may accelerate or promote ALD by increasing hepatic homocysteine and SAH concentrations; decreasing hepatic SAM and glutathione concentrations and the SAM-SAHC ratio; increasing cytochrome P4502E1 activation and lipid peroxidation; up-regulating endoplasmic reticulum stress markers, including sterol regulatory element–binding protein-1, and proapoptotic gene caspase-12; and decreasing global DNA methylation. Betaine may attenuate ALD by increasing the synthesis of SAM and, eventually, glutathione, decreasing the hepatic concentrations of homocysteine and SAH, and increasing the SAM-SAHC ratio, which can trigger a cascade of events that lead to the activation of phosphorylatedethanolamine methyltransferase, increased phosphorylcholine synthesis, and formation of VLDL for the export of triacylglycerol from the liver to the circulation. Additionally, decreased concentrations of homocysteine can down-regulate endoplasmic reticulum stress, which leads to the attenuation of apoptosis and fatty acid synthesis. Am J Clin Nutr 2007;86:14–24.

KEY WORDS Alcohol, betaine, S-adenosylmethionine, folate, liver disease

INTRODUCTION
Alcoholic liver disease (ALD) is characterized by fatty liver, steatohepatitis, fibrosis, cirrhosis, and potentially hepatocellular carcinoma. Several mechanisms have been proposed for the pathogenesis of ALD, including acetaldehyde toxicity, oxidative stress, endotoxins, cytokines, chemokines, a compromised immune system, and nutritional deficiencies. Increasing evidence suggests that altered methionine folate metabolism can also contribute to the development of ALD (1, 2). Chronic ethanol exposure has been shown to decrease hepatic concentrations of S-adenosylmethionine (SAM) (3–5), increase plasma concentrations of homocysteine (6–8), increase hepatic concentrations of S-adenosylhomocysteine (SAH) (9–11), and decrease plasma concentrations of folate (12) in animal and human studies. These changes in methionine metabolism are associated with different degrees of liver injury. Conversely, exogenous administration of SAM has been shown to attenuate alcoholic liver injury in animal studies (3, 5, 13). In addition, betaine (trimethylglycine), a metabolite of choline, has been shown to attenuate alcoholic liver injury by increasing the concentrations of hepatic SAM and decreasing the concentrations of homocysteine and SAH in other animal studies (8, 11, 14, 15). Understanding the role of SAM, folate, and betaine in mitigating alcoholic liver injury may help to develop effective and safe therapies for ALD and non-ALD.

The National Institute on Alcohol Abuse and Alcoholism, in collaboration with the Office of Dietary Supplements and National Center for Complementary and Alternative Medicine of the National Institutes of Health, organized a symposium on the role of SAM, betaine, and folate in the treatment of ALD in Bethesda, MD, held on 3 October 2005. The following topics were discussed by 7 speakers: 1) methionine metabolism (Norlin...
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J Benevenga, 2) methionine adenosyltransferase (MAT) and SAM in liver health and disease (Shelly C Lu), 3) effects of folate metabolism on ALD (Charles H Halsted), 4) SAM and betaine therapy in ALD (Craig J McClain and Shirish Barve), 5) mechanism of action of betaine in the treatment of liver injury (Kusum K Kharbanda), 6) betaine in alcohol-induced hyperhomocysteinemia and endoplasmic reticulum (ER) stress (Neil Kaplowitz), and 7) betaine and SAM therapy in nonalcoholic steatohepatitis (NASH) (Manal F Abdelmalek). The following is a summary of the symposium.

METHionine METABOLISM CYCLE

Methionine is an essential amino acid that is primarily metabolized in the liver (Figure 1). The first step in methionine metabolism is the formation of SAM in a reaction catalyzed by MAT (16, 17). Under normal conditions, most of the SAM generated is used in transmethylation reactions, whereby SAM is converted to SAH by transferring the methyl group to diverse biological acceptors (16). SAH is then converted to homocysteine and adenosine in a reversible reaction catalyzed by SAH hydrolase (16). In the liver, homocysteine is metabolized by transsulfuration and transmethylation pathways. In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine in a reaction catalyzed by cystathionine β-synthase, which requires vitamin B-6 as a cofactor. The activity of cystathionine β-synthase is allosterically regulated by SAM (18, 19). Cystathionine is then cleaved by another vitamin B-6–dependent enzyme, γ-cystathionase, which results in the release of free cysteine, the rate-limiting precursor for reduced glutathione synthesis (16, 20). In the transmethylation pathway, homocysteine can be converted to methionine by 2 alternate reactions (16, 17). Normally, utilizing folate and through the action of methionine synthetase (MS), a methyl group is transferred from N5-, N10-methylenetetrahydrofolate (MTHF) to vitamin B-12 to form methylcobalamin, which in turn transfers the methyl group to homocysteine to produce methionine. Alternatively, when MS is compromised by exposure to ethanol (9, 10, 14), a methyl group is transferred from betaine to homocysteine to form methionine in a reaction catalyzed by betaine-homocysteine methyltransferase (BHMT). The conversion of homocysteine to methionine is an essential reaction to conserve methionine, detoxify homocysteine, and produce SAM.

ROLE OF S-ADENOSYLMETHIONINE IN ALCOHOLIC LIVER DISEASE

SAM, a metabolite of methionine, is an important molecule that is required for many vital functions and survival of cells in the body. It is the principal biological methyl donor required for methylation of DNA, RNA, biogenic amines, phospholipids, histones, and other proteins. It is the precursor for the synthesis of polyamines, which are required for cell proliferation and the maintenance of cell viability. In the liver, SAM is a precursor for glutathione—a major endogenous antioxidant that protects cells against injury by scavenging free radicals, which are involved in the pathogenesis of ALD. Thus, SAM deficiency can impair many vital functions of the liver, which render it susceptible to injury by toxic agents such as alcohol.

Ethanol, hepatic SAM depletion, and consequences

Animal and human studies suggest a link between ethanol consumption and hepatic SAM depletion. Chronic ethanol administration depleted the hepatic concentrations of SAM in rats (4, 21–23), in mice (5, 24), in baboons (3), and in micropigs (10, 25). Reduced hepatic SAM concentrations have also been reported in alcoholic hepatitis patients (26).

Hepatic SAM depletion by chronic ethanol administration is associated with liver injury of variable magnitude: fatty liver in rats (4, 21, 23); fatty liver, inflammation, and fibrosis in baboons (3); fatty liver and inflammation in micropigs (25); and hepatitis in humans (26). The effect of SAM depletion is well characterized in MAT1A knockout mice, which have markedly elevated serum methionine concentrations and reduced hepatic SAM and glutathione concentrations (27). At 3 mo, MAT1A knockout mice develop hepatic hyperplasia and are more prone to develop fatty liver due to a choline-deficient diet. At 8 mo of age, these
mice spontaneously develop NASH (27), and by 18 mo, the majority of the knockout mice develop hepatocellular carcinoma even when consuming a normal diet (28).

Mechanisms of alcohol-induced hepatic SAM depletion

Several mechanisms have been proposed for SAM depletion: 1) inactivation of MAT, 2) excessive consumption of SAM by liver, and 3) inhibition of endogenous methionine synthesis due to impaired homocysteine methylation.

Inactivation of MAT impairs the metabolism of methionine to SAM, which leads to SAM depletion. For instance, MAT1A knockout mice have reduced hepatic concentrations of SAM and glutathione (27). Chronic alcohol exposure may decrease hepatic SAM concentrations by inactivating MAT. The activity of MAT was significantly reduced in liver biopsy samples from alcoholic and nonalcoholic cirrhotic patients (29, 30). This effect could be partly due to a decreased expression of MAT. Indeed, recent data indicate a 50% decrease in the expression of MAT1A (liver-specific MAT) in liver samples obtained from alcoholic hepatitis patients (26) and from ethanol-fed microcrops (31). The decrease in MAT activity may occur pre- and posttransitionally (17). MAT1A expression is diminished in end-stage cirrhotic patients independent of the etiology (32). MAT I/III (but not MAT II) can also be inactivated via covalent modification of a critical cysteine residue at position 121 (17). The inactivation can be reversed by glutathione and other thiol-reducing agents. Nitrosylation of Cys 121 of MAT I/III and its inactivation have been shown, both in vitro and in vivo, in animals treated with lipopolysaccharide (LPS) (33, 34). Ethanol metabolism is known to generate free radicals, both reactive oxygen species and reactive nitrogen species, which may inactivate hepatic MAT through the oxidation or nitrosylation of cysteine residue at position 121.

Ethanol may deplete hepatic SAM concentrations by increased SAM consumption. This was apparent in a rat study in which chronic alcohol administration decreased hepatic SAM and glutathione concentrations without affecting MAT activity (23). This suggests that the utilization of SAM is increased as a precursor for the synthesis of glutathione to counteract alcohol-induced oxidative stress.

Chronic ethanol administration has been shown to decrease the hepatic activity of MS and to reduce hepatic concentrations of folate and betaine (10, 12, 14, 31, 35). These factors are known to participate in the synthesis of endogenous methionine through methylation of homocysteine. Thus, alcohol may deplete hepatic SAM by inhibiting the synthesis of its endogenous precursor methionine.

SAM treatment in alcoholic liver injury

The fact that SAM is essential for multiple metabolic reactions and that chronic ethanol administration can deplete its hepatic concentration in association with liver injury prompted researchers to evaluate its role in the attenuation of ALD in animals and humans.

In intact rats, SAM administration attenuated alcohol-induced steatosis and restored hepatic glutathione concentrations (13), whereas it attenuated ethanol-induced depletion of mitochondrial glutathione and restored mitochondrial function in hepatocytes (36). In isolated perfused rat liver, SAM administration attenuated ethanol hepatotoxicity by 1) decreasing aspartate transaminase (AST) and lactate dehydrogenase release in the perfusate, 2) restoring mitochondrial and homogenate glutathione concentrations, and 3) restoring normal hepatic oxygen consumption (37). In mice, SAM treatment significantly attenuated acute alcohol-induced liver injury [steatosis, necrosis, and increased serum alanine transaminase (ALT) activity], which was associated with restoration of hepatic SAM and mitochondrial glutathione concentrations and attenuation of lipid peroxidation (5). In baboons, SAM attenuated alcohol-induced liver injury by repairing mitochondrial injury, which restored plasma glutathione concentrations and decreased plasma concentrations of AST (3).

The therapeutic potential of SAM was tested in a 24-mo randomized, placebo-controlled, double-blind, multicenter clinical trial in 123 patients with alcoholic cirrhosis. SAM treatment improved survival or delayed the need for liver transplantation in patients with alcoholic liver cirrhosis, especially in those with less advanced liver disease (38). In this trial, increased hepatic concentrations of glutathione may have contributed to the beneficial effect of SAM because, in another study, oral administration of 1.2 g SAM/d for 6 mo significantly increased hepatic glutathione concentrations in ALD patients (39). In a recent clinical meta-analysis review, Rambaldi and Gluud (40) could not find evidence to support or refute the use of SAM in the treatment of patients with ALD.

SAM treatment in non-alcoholic liver injury

SAM has also been shown to attenuate liver injury induced by other toxic agents such as CCl₄, acetalaminophen, and diethylnitrosamine. In rats, SAM treatment attenuated CCl₄-induced liver fibrosis by restoring hepatic MAT activity and glutathione concentrations and by reducing lipid peroxidation (41, 42). In cultured hepatocytes, SAM prevented CCl₄-induced hepatotoxicity by suppressing the leakage of glutamate-oxalate-transaminase and glutamate-pyruvate-transaminase (43). In acetalaminophen-treated mice, SAM significantly attenuated liver injury by preventing decreases in liver and blood SAM concentrations and by attenuating both cytosolic and mitochondrial glutathione depletion and mitochondrial dysfunction (44). In addition, SAM appears to have protective effects against hepatic carcinogenesis. SAM inhibited growth and induced phenotypic reversion and apoptosis of preneoplastic cells and decreased the development of hepatic diethylnitrosamine-induced neoplastic nodules in rats (45, 46). These changes were associated with restoration of the hepatic SAM pool and DNA methylation. Furthermore, exogenous SAM inhibits the growth of cultured hepatoma cells (47).

Mechanisms of SAM’s protective effects

SAM may provide protection against liver injuries through various mechanisms, and some of these mechanisms are discussed below.

Attenuation of oxidative stress by restoring glutathione concentrations

Oxidative stress plays a major role in the development of alcoholic liver injury. This can occur because of excess accumulation of free radicals, their delayed elimination, or both. Glutathione, an endogenous antioxidant, is capable of attenuating oxidative stress by scavenging free radicals. Therefore, one way to attenuate alcoholic liver injury is to increase hepatic concentrations of glutathione. In this regard, SAM administration restored
methylthioadenosine (MTA), a metabolite of SAM, suppressed RAW 264.7 murine monocyte cell line (50), and injury by decreasing TNF-α concentrations. These results suggest that SAM may attenuate alcoholic liver injury by decreasing TNF-α concentrations and increasing IL-10 concentrations. The inhibitory effect of SAM on TNF-α expression most probably occurs at the transcriptional level. Work done in murine macrophages has shown that SAM and its metabolite MTA protected rat hepatocytes from okadaic acid-induced apoptosis in a dose-dependent manner. This effect was mediated through attenuation of mitochondrial cytochrome-c release, caspase-3 activation, and poly(ADP-ribose) polymerase cleavage (55). SAM treatment also inhibited bile acid–induced apoptosis of cultured rat hepatocytes (56, 57). Thus, SAM may attenuate ALD by preventing apoptosis of hepatocytes.

The mechanism of this effect is targeted at the mitochondria because SAM prevented okadaic acid–induced cytochrome-c release. However, the molecular mechanism of this effect remains unclear.

**Induction of apoptosis of liver tumor cells**

Chronic alcohol consumption is a risk factor for hepatocellular carcinoma in humans. In a rat study, chronic ethanol administration significantly decreased hepatic concentrations of methionine, SAM, and DNA methylation by ∼40% (58). In addition, c-myc was hypomethylated and its mRNA concentration increased, and genome-wide DNA strand breakage increased. Studies in the ethanol-fed micropig model showed an association of a decrease in the ratio of SAM to SAH (SAM:SAH) with hepatocellular apoptosis (10) and with increased DNA oxidation and strand breaks (25). These changes may predispose the liver to malignancy, which suggests a role of ethanol in the development of hepatocellular carcinoma. Apoptosis is a mechanism by which tumor cells can be eliminated. In this regard, SAM has been shown to induce apoptosis in the liver cancer cell lines HepG2 and HuH-7 (55). SAM induced apoptosis of preneoplastic cells in rats induced by diethylamionamine (45, 46). Furthermore, SAM has been shown to selectively induce an apoptotic factor, Bcl-xS, in a time- and dose-dependent manner in HepG2 cells, but not in normal hepatocytes, by increasing alternative splicing of Bcl-x (59), which suggests a mechanism whereby SAM can induce the apoptosis of neoplastic cells.

**SAM summary**

SAM supplementation may attenuate ALD through various mechanisms (Figure 2). By being a precursor of glutathione as well as by activating cystathionine β-synthase, SAM up-regulates the trans-sulfuration pathway, which leads to the increased synthesis of glutathione, which in turn attenuates oxidative stress.

The down-regulation of oxidative stress is expected to attenuate inflammation, fibrosis, and eventually ALD. SAM may attenuate inflammation and, thus, ALD by down-regulating TNF-α and up-regulating IL-10 synthesis. SAM also may attenuate liver injury by inhibiting the apoptosis of normal hepatocytes as well as by stimulating the apoptosis of liver cancer cells.

**ROLE OF FOLATE IN ALCOHOLIC LIVER DISEASE**

Folate is a water-soluble vitamin that plays an integral role in methionine metabolism and DNA synthesis. Folate in its 5-methyltetrahydrofolate (5-MTHF) form can transfer a methyl group to homocysteine via an MS-catalyzed reaction to form endogenous methionine, which is a precursor of SAM. Thus, folate helps maintain normal concentrations of homocysteine, methionine, and SAM. Folate deficiency can impair methionine metabolism, which leads to hyperhomocysteinemia as well as depletion of methionine and SAM, which are important features of ALD (60).

**Folate concentrations in alcoholics**

Studies performed in the United States before folic acid fortification and more recently in several European countries have reported decreased serum or red blood cell folate concentrations in the majority of chronic alcoholic patients who consume >80 g ethanol/d (6, 12, 61, 62). More than 40 y ago, a US study
reported a greater incidence of very low serum folate concentrations (<3.0 ng/mL) in patients with ALD than in alcoholics without liver disease (61), whereas another survey found low serum folate concentrations in 78% of 140 ALD patients admitted to a large US city hospital (63).

Mechanisms of folate depletion in alcoholics

The possible causes of folate deficiency in chronic alcoholism include: 1) diet lacking in folate-rich foods (61), 2) intestinal malabsorption (64) that may be due to decreased transcription of the reduced folate carrier required for folate transport across intestinal membranes (65), 3) decreased liver uptake (66, 67), and 4) increased urinary excretion (68–70). Decreased liver folate storage may be a critical cause of folate deficiency in ALD patients.

Folate deficiency and homocysteine concentrations in alcoholics

Increasing evidence suggests that homocysteine is involved in the pathogenesis of alcoholic liver injury (71). Because folate is required for the metabolism of homocysteine, its deficiency can lead to the elevation of serum homocysteine concentrations. Indeed, folate deficiency was shown to be associated with elevated serum homocysteine concentrations in 50–60% of chronic alcoholics in Spanish (12) and Portuguese (6) studies. The Spanish study of 103 heavy drinkers included 19 ALD patients whose mean serum homocysteine concentration was not different from that of the alcoholics without liver disease. In these subjects, elevated serum homocysteine concentration was highest in a subgroup of patients who carried the methyltetrahydrofolate reductase (MTHFR) 677T->T genotype (12). MTHFR converts 5,10-methylene tetrahydrofolate to methyltetrahydrofolate, the substrate for MS. Because 5,10-methylene tetrahydrofolate is also a substrate for thymidine synthase, which converts uracil to thymidine, its diversion to the MTHFR reaction results in less availability of uracil for the thymidine synthase reaction, with subsequent decrease in thymidine synthesis and hence nucleotide imbalance that may contribute to an increased risk of carcinogenesis (10). Serum homocysteine concentrations were elevated in >80% of a series of 42 chronic alcoholics admitted to a Swedish detoxification center, and these concentrations declined rapidly with abstinence and a nutritious diet (72).

In addition to depleting folate concentrations, chronic ethanol exposure decreases the activity of MS (9, 10, 14, 31, 73), which is required to catalyze the transfer of a methyl group from folate to homocysteine to form methionine. Thus, chronic ethanol can elevate plasma homocysteine concentrations by simultaneously affecting folate concentrations and MS activity.

Role of dietary folate deficiency in the development of alcoholic liver injury

The micropig has been used as a model to understand the role of folate deficiency in the development of ALD (25). In this study, 4 groups of 6 animals each were administered the following diets for 14 wk: folate-sufficient diet (control), folate-deficient diet, ethanol-containing (40% of total kcal) folate-sufficient diet, and ethanol-containing folate-deficient diet. The effects of diets were determined by evaluating histopathologic changes, alterations in methionine metabolism, and markers of oxidative stress. After 3 mo of feeding, serum homocysteine concentrations were increased maximally in the combined folate-deficient ethanol diet group together with an 8-fold increase in serum AST and the histopathology of steatohepatitis (25). In contrast, 12 mo of ethanol feeding was required for induction of similar histopathology in micropigs fed ethanol with a folate-sufficient diet (74). These findings were associated with elevated hepatic SAH concentrations, reduced SAM:SAH and

FIGURE 2. Proposed mechanisms for the role of S-adenosylmethionine (SAM) in alcoholic liver disease (ALD). SAM supplementation may attenuate ALD through various mechanisms. By being a precursor of glutathione (GSH) and by activating cystathionine β-synthase, SAM up-regulates the trans-sulfuration pathway, which leads to the increased synthesis of GSH, which in turn attenuates oxidative stress. The down-regulation of oxidative stress is expected to attenuate inflammation, fibrosis, and eventually ALD. SAM may attenuate inflammation and thus ALD by down-regulating tumor necrosis factor-α (TNF-α) and up-regulating interleukin-10 (IL-10) synthesis. SAM also may attenuate liver injury by inhibiting the apoptosis of normal hepatocytes and by stimulating the apoptosis of liver cancer cells.
SAM and glutathione concentrations, decreased global DNA methylation (25), increased DNA strand breaks, and increased concentrations of hepatic lipid peroxide, malondialdehyde, and the DNA oxidation product 8-oxo-2′-deoxyguanosine. It is important to note that folate deficiency alone did not affect liver histology, whereas ethanol alone induced only steatosis in some animals.

Using liver specimens from the same groups of micropigs, studies of transmethylation regulatory enzymes found that folate deficiency or ethanol exposure, singly or in combination, reduced concentrations of MTHFR, MS, MAT, and SAH hydrolase (31). A subsequent study of molecular mechanisms using liver samples from the same animals found that hepatocellular apoptosis was maximal in pigs fed the combined folate-deficient and ethanol diet. This finding was associated with increased transcription and protein concentrations of CYP2E1 and activation of ER stress markers, including sterol regulatory element–binding protein (SREBP), lipid synthesis enzymes, and activated caspase-12 (75). The findings of correlations of concentrations of CYP2E1, apoptosis, and ER stress signals with elevated concentrations of homocysteine and SAH and decreases in SAM: SAH were consistent with the notion that the pathogenesis of ALD is mediated through effects of ethanol feeding, magnified by folate deficiency, on methionine metabolism.

It is important to note that, with folic acid fortification of the American diet, chronic ethanol exposure may not deplete hepatic folate to an extent sufficient to impair homocysteine metabolism, although ethanol may still impair homocysteine metabolism by decreasing the activity of MS. This notion is corroborated by a study in which 4 wk of ethanol feeding of rats with 20 times the basal folate requirement did not reduce plasma or hepatic folate concentrations, although plasma homocysteine concentrations were significantly elevated (7).

In summary, in the absence of dietary fortification with folic acid, folate deficiency and the accompanying hyperhomocysteinemia are common findings in chronic alcoholics who consume >80 g ethanol/d. ALD patients are probably at greater risk of folate deficiency because of the decreased liver storage of folate. Studies using the micropig model have shown that the onset of ALD is accelerated in the presence of folate deficiency, which also magnifies the effect of chronic ethanol exposure on altered methionine metabolism. A significant role of altered methionine metabolism in the pathogenesis of ALD is supported by recent studies that have linked the induction of elevated SAH and homocysteine concentrations to increased activation of CYP2E1 and ER stress pathways of apoptosis and steatosis in folate-deficient ethanol-fed micropigs. Folate deficiency alone does not lead to liver injury, but it can accentuate or promote the development of ALD. Whether exogenous folate administration would attenuate ALD needs further investigation.

### ROLE OF BETAINES IN ALCOHOLIC LIVER DISEASE

Betaine (trimethylglycine) is an important human nutrient obtained from a variety of foods, and it is also available as a dietary supplement. As a methyl donor, betaine provides a methyl group to homocysteine to form methionine in a reaction that is catalyzed in the liver by BHMT. This helps to maintain an adequate supply of liver methionine for the synthesis of SAM and the regulation of the homocysteine concentration. Betaine is synthesized in the liver from choline in a reaction that is catalyzed by choline oxidase. To understand the role of betaine in the treatment of ALD, researchers have used various animal models of alcoholic liver injury, which are described below.

### Rat model of alcoholic fatty liver injury and betaine

In this model, animals are fed a Lieber-DeCarli ethanol-containing diet (36% of total energy) for up to 4 wk. This treatment results in the development of fatty liver, which is associated with significant alterations in methionine metabolism. The liver injury does not progress to necroinflammation or fibrosis despite continuous ethanol feeding. In this model, betaine treatment attenuates fatty liver and restores (normalizes) methionine metabolism. These protective effects of betaine are presented below in detail.

**Betaine attenuates fatty liver**

Using the rat model of alcoholic fatty liver described above, researchers showed that feeding a diet containing 0.5% betaine for 2–4 wk can attenuate ethanol-induced accumulation of triacylglycerol in the liver (4, 14, 21). In addition, histopathologic evaluation confirmed that betaine attenuated alcoholic fatty liver. These studies also showed that long-term ethanol feeding can deplete endogenous hepatic betaine concentrations, whereas dietary supplementation of betaine can elevate its hepatic concentrations in both control and ethanol-fed rats. Subsequent studies by these investigators showed that betaine can also reverse ethanol-induced fatty liver despite continued feeding of ethanol (76). Taken together, these studies suggest that dietary betaine can be absorbed from the gastrointestinal tract and transported to the liver, where it can attenuate alcoholic fatty liver.

**Betaine lowers homocysteine concentrations**

Alcoholics have elevated concentrations of plasma homocysteine (6, 72), and chronic ethanol administration elevates total plasma homocysteine concentrations in rats (7, 77) and in micropigs (10, 25). Because betaine is known to methylate homocysteine to form methionine, the effect of betaine on the ethanol-induced release of homocysteine was determined in vitro (15). In this study, hepatocytes isolated from rats fed the Lieber-DeCarli control or ethanol diet for 4 wk were incubated in vitro for 4 h, and the concentration of homocysteine was measured in media as a reflection of cellular concentrations. The hepatocytes from ethanol-fed rats were found to release twice as much homocysteine into the media as controls. Furthermore, the addition of methionine to the incubation mixtures of control and ethanol-fed hepatocytes resulted in a marked increase in homocysteine generation in both cell types (15). Betaine supplementation in the incubation medium prevented the increases in homocysteine by methionine-treated control cells as well as by the cells from ethanol-treated rats. The inhibiting effect of betaine on the release of homocysteine from hepatocytes was recently confirmed by the same group of investigators (78); however, in this study, SAM failed to inhibit the release of homocysteine. Because homocysteine concentrations are associated with the development of fatty liver, betaine may attenuate alcoholic fatty liver, at least in part by preventing the intrahepatic accumulation of homocysteine through its methylation to form methionine. In another study, betaine supplementation increased hepatic BHMT activity in the control animals, which was further increased in the ethanol-fed rats (14). Thus, dietary betaine appears to promote...
the metabolism of homocysteine by providing a methyl group as well as by increasing the activity of BHMT, which catalyzes the transfer of a methyl group from betaine to homocysteine.

**Betaine attenuates SAH concentrations**

SAH is formed when SAM transfers its methyl group to various compounds, catalyzed by many different methyltransferases. SAH is further metabolized to homocysteine and adenosine through a reaction catalyzed by SAH hydrolase. This reaction is reversible, and the generation of SAH from homocysteine is thermodynamically favored over the synthesis of homocysteine. The reaction proceeds toward homocysteine synthesis only when the products (homocysteine and adenosine) are removed by further metabolism. However, if the products are allowed to accumulate, the hepatic concentrations of SAH can be elevated, which could inhibit the activities of many SAM-dependent methyltransferases.

Chronic ethanol feeding has been shown to increase hepatic concentrations of SAH in rats (9), mice (24), and microops (10, 25). Furthermore, hepatocytes obtained from ethanol-fed rats showed a significant 2-fold increase in SAH concentrations, which were further elevated when the hepatocytes were incubated with methionine (11). When betaine was added to the incubation medium, the concentrations of SAH were significantly reduced. In another study, betaine was shown to attenuate adenosine-induced increases in SAH concentrations in isolated hepatocytes from rat liver (79). Thus, betaine may prevent alcoholic fatty liver, at least in part, by attenuating SAH production.

Researchers further investigated whether betaine could attenuate SAH-induced hepatocyte apoptosis. Various concentrations of adenosine were used to increase intracellular concentrations of SAH in cultured rat hepatocytes. Adenosine-induced increases in SAH concentrations were associated with increases in caspase-3 activity and DNA fragmentation, both of which are markers of apoptosis (79). The addition of betaine to the incubation medium significantly attenuated adenosine-induced caspase-3 activity and DNA fragmentation by attenuating the adenosine-induced increases in SAH concentrations. These results were further corroborated by using tubercidin, a potent inhibitor of SAH hydrolase, which has also been used to increase intracellular concentrations of SAH (80). Betaine could also protect tubercidin-induced hepatocyte apoptosis. The results of the studies obtained by using adenosine and tubercidin in vitro indicate that betaine may prevent alcoholic liver injury by attenuating the SAH-induced apoptosis of hepatocytes. Inhibition of the critical methyltransferase (such as isoprenyl cysteine methyltransferase) by elevated intracellular SAH concentrations may be responsible for the adenosine- or tubercidin-induced apoptosis in hepatocytes (79, 80). Recent studies have shown that the carboxyl methylation reaction of small GTPases is a crucial activation step that facilitates these proteins to participate in anti-apoptotic signaling pathways (81). Although increased homocysteine concentrations can induce ER stress and cause caspase activation, homocysteine concentrations are not increased by adenosine exposure (79).

**Betaine elevates hepatic SAM concentrations**

Because betaine can methylate homocysteine to form methionine, which is a precursor of SAM, it is logical to assume that dietary betaine supplementation would affect hepatic concentrations of SAM, which may in turn attenuate liver injury. In a rat model of alcoholic fatty liver, betaine administration for 4 wk increased the hepatic concentrations of SAM 2-fold in control animals and 4-fold in the ethanol-fed rats (4, 14). The higher concentrations of SAM were associated with attenuated fatty liver in ethanol-fed rats. Another study showed that betaine administration, even for a short period (2 wk) generated increased hepatic concentrations of SAM in both control and ethanol-fed rats and significantly lower ethanol-induced accumulation of hepatic triacylglycerol (21). The stimulatory effect of betaine on SAM production was further confirmed in an in vitro study in which betaine significantly increased the concentrations of SAM in isolated hepatocytes from both control and ethanol-treated rats (11). These studies suggest that betaine may attenuate alcoholic fatty liver by increasing SAM production in the liver.

**Betaine elevates SAM:SAH**

Whereas SAM is the major source of methyl groups required for the methylation of many compounds in the liver, SAH is a competitive inhibitor for many SAM-dependent methyltransferases because it acts on the same site as SAM on these enzymes. The potential pathogenicity of SAH lies in its high affinity binding to the catalytic region of most SAM-dependent methyltransferases, which enables it to act as a potent product inhibitor. The $K_i$ value for SAH is often less than the $K_m$ value for SAM for many of the methyltransferases (82). Therefore, the ratio of SAM to SAH in cells appears to be the prime regulator of the activities of most methyltransferases, and any significant decrease in the ratio will negatively affect methylation reactions.

Chronic ethanol exposure is associated with increased hepatic concentrations of SAH and decreased hepatic concentrations of SAM. This effect of ethanol is expected to decrease the SAM:SAH ratio, which can inhibit the activities of many SAM-dependent methyltransferases and may contribute to alcoholic fatty liver. Researchers have investigated the role of betaine in the correction of the SAM:SAH altered by ethanol. In one study, the intracellular SAM:SAH in isolated hepatocytes from ethanol-fed rats was significantly lower than that from control rats. The addition of betaine to the incubation medium significantly increased the SAM:SAH in hepatocytes from both the control and ethanol-fed rats (11). These results were confirmed in a recent study in which researchers compared the potencies of betaine and SAM supplementation in increasing the intracellular SAM:SAH ratio in the hepatocytes from ethanol-fed and pair-fed control rats (78). Supplementation of betaine or SAM in the incubation media increased this ratio in hepatocytes from both the control and ethanol-fed rats and attenuated the ethanol-induced increase in hepatocellular triacylglycerol concentrations by $\approx20\%$. Although the effects of both compounds in enhancing the SAM:SAH were similar, the mechanisms of their effects appear to be different. Betaine is likely to increase the SAM:SAH by lowering intracellular SAM concentrations as a result of lowering homocysteine concentrations and by increasing SAM concentrations via increases in methionine concentrations through the activity of BHMT. On the other hand, SAM supplementation is likely to increase the SAM:SAH by increasing intracellular SAM concentrations. The difference in the mechanisms is due to the fact that betaine, but not SAM, can effectively prevent the accumulation of homocysteine via its methylation and subsequent formation of methionine and then SAM. These results suggest that betaine may attenuate alcoholic liver injury by correcting the SAM:SAH altered by ethanol exposure.
**SAM:SAH and phosphatidylethanolamine methyltransferase activity**

Phosphatidylethanolamine methyltransferase (PEMT) catalyzes the methylation of phosphatidylethanolamine (PE) to form phosphatidylcholine (PC), where SAM acts as a methyl donor. Although PC can also be synthesized via the Kennedy pathway in the liver, which accounts for ≈60–70% of PC synthesized in the liver (83), it was recently shown that the PC synthesized by the PEMT pathway is an important and essential constituent for the synthesis and secretion of VLDL, which is required for the export of liver triacylglycerol (84). It was recently shown that PEMT knockout mice spontaneously develop steatosis, despite the ingestion of the recommended dietary intake of choline (85).

To determine the effect of an altered SAM:SAH on PEMT activity, hepatic microsomal fractions were incubated with a constant amount of SAM and different amounts of SAH so that the SAM:SAH was equal to either 5.0 or 2.5 to correspond to the ratios seen in the livers of control-fed or ethanol-fed rats, respectively. The PEMT activity at an SAM:SAH of 2.5 was only 50% of that observed at an SAM:SAH of 5.0 (11). These researchers also showed that ethanol significantly inhibits the conversion of PE to PC in isolated hepatocytes (86), which suggests that this effect of ethanol is mediated through the inhibition of PEMT activity. Taken together, these results support the notion that the decrease in PEMT activity due to the decreased intracellular SAM:SAH seen in hepatocytes of ethanol-fed rats can inhibit PC synthesis. A reduced amount of PC can lead to defective synthesis and secretion of VLDL, which may be partly responsible for the hepatic steatosis seen in these rats. Because betaine has been shown to elevate the SAM:SAH, this may be a mechanism by which betaine restores PEMT activity, elevates PC concentrations, normalizes VLDL secretion, and attenuates alcoholic fatty liver.

**Mouse model of alcoholic liver injury (fatty liver and necroinflammation) and betaine**

The intragastric ethanol-fed mouse model of alcoholic liver injury was used to further understand the role of betaine in the treatment of ALD (8, 71, 87). In this model, 4 wk of ethanol feeding lead to histopathologic features of early ALD, namely prominent steatosis, accompanied by necroinflammatory foci and scattered apoptosis of hepatocytes. These histopathologic changes are associated with an elevated ER stress response, as indicated by the up-regulation of the proapoptotic gene GADD153 and of the lipogenic transcription factor SREBP-1. The mechanisms of ER stress in this model are not clear. In addition, this model showed increased plasma concentrations of ALT and increased hepatic mRNA concentrations of TNF-α, CD14, and CYP2E1. Furthermore, this model showed many features of altered methionine metabolism, including several-fold increases in plasma homocysteine concentrations, decreased hepatic SAM concentrations, and decreased hepatic mRNA concentrations of MS and BHMT. In this model, betaine feeding attenuated the pathologic features of alcoholic liver injury (fatty liver, necroinflammation, and apoptosis) and decreased plasma ALT concentrations.

These features were associated with attenuated plasma homocysteine concentrations, increased hepatic SAH concentrations, several-fold increased hepatic SAM concentrations, and several-fold increased SAM:SAH ratios (8, 71, 87). In addition, betaine attenuated the alcohol-induced ER stress response as shown by the down-regulation of proapoptotic gene GADD153 and of lipogenic transcription factor SREBP-1. Betaine feeding did not abolish the induction of CYP2E1 by ethanol, nor did it attenuate ethanol-induced increases in hepatic TNF-α or CD14 mRNA. These results suggest that betaine attenuates alcoholic liver injury by decreasing homocysteine concentrations, increasing SAM concentrations, and increasing the SAM:SAH in the liver. This beneficial effect of betaine does not appear to be mediated through the gene expression of CYP2E1, TNF-α, or CD14.

**Rat model of alcoholic liver fibrosis and betaine**

To understand the role of betaine in the treatment of liver fibrosis, researchers have used a rat model of ethanol plus CCl4-induced liver fibrosis (88). In this model, administration of ethanol in drinking water and a low dose of CCl4 results in liver fibrosis, which is associated with hepatic lipid peroxidation and increases in plasma concentrations of AST and ALT. Betaine treatment prevented liver fibrosis, attenuated lipid peroxidation, and decreased plasma transaminase activities.

**Nonalcoholic steatohepatitis and betaine**

The role of betaine in the treatment of NASH has been evaluated in 3 human studies. In a prospective, randomized, double-blind therapeutic trial (n = 191 patients), oral administration of betaine glucuronate for 8 wk reduced hepatic steatosis by 25%, reduced hepatomegaly by 8%, and significantly attenuated the hepatic concentrations of AST, ALT, and γ-glutamyl transferase in NASH patients (89). In addition, betaine treatment significantly improved discomfort in the abdominal upper right quadrant.

In a pilot study of 10 subjects with NASH, 7 of 10 patients completed 1 y of treatment with betaine. A significant improvement in serum concentrations of AST (P = 0.02) and ALT (P = 0.007) occurred during treatment. Aminotransferases normalized in 3 of 7 patients decreased by >50% in 3 of 7 patients and remained unchanged in one patient when compared with baseline values. A marked improvement in serum concentrations of aminotransferases (ALT, −39%; AST, −38%) also occurred during treatment in those patients who did not complete 1 y of treatment. Similarly, a marked improvement in the degree of steatosis, necroinflammatory grade, and stage of fibrosis was noted at 1 y of treatment with betaine (90). In an ongoing study of patients affected with NASH, betaine treatment attenuated serum ALT concentrations and improved the grades of steatosis, inflammation, and fibrosis (91).

**Betaine summary**

Dietary betaine can be absorbed from the intestine and transported to the liver, which leads to increased hepatic concentrations of betaine (Figure 3). In the liver, betaine can transfer its one methyl group to homocysteine to form methionine. This can result in decreased concentrations of homocysteine and increased concentrations of methione in the liver. Consequently, the former results in decreased hepatic concentrations of SAH, whereas the latter can increase hepatic SAM concentrations, which leads to an increased SAM:SAH. An elevated SAM:SAH can trigger a cascade of events leading to PEMT activation, PC synthesis, formation of proper VLDL, export of triacylglycerol, and attenuation of fatty liver. Decreased hepatic concentrations...
of homocysteine can attenuate ER stress, which may result in decreased hepatic concentrations of homocysteine and increased concentrations of methionine in the liver. Consequently, the former results in decreased hepatic concentrations of S-adenosylhomocysteine (SAH), whereas the latter can increase hepatic S-adenosylmethionine (SAM) concentrations, which leads to an elevated ratio of SAM to SAH (SAM:SAH). An elevated SAM:SAH can trigger a cascade of events that leads to phosphatidylethanolamine methyltransferase (PEMT) activation, phosphatidylcholine (PC) synthesis, the formation of proper VLDL, the export of triacylglycerol, and the attenuation of fatty liver. Decreased hepatic concentrations of homocysteine can attenuate endoplasmic reticulum (ER) stress, which may result in the down-regulation of proapoptotic genes and consequently in the attenuation of apoptosis, inflammation, and fibrosis. The down-regulation of another ER stress gene, sterol regulatory element–binding protein, can reduce hepatic fatty acid synthesis, which may result in a reduction in fatty liver. Increased hepatic concentrations of SAM can activate cystathionine β-synthase, which leads to the up-regulation of the trans-sulfuration pathway, the increased synthesis of glutathione (GSH), and the attenuation of oxidative stress. Thus, betaine can ameliorate ALD by attenuating fatty liver, inflammation, and fibrosis.

OVERALL SUMMARY

It is evident that altered methionine metabolism can contribute to the development of ALD. Furthermore, information available from in vitro and animal studies clearly suggests that SAM and betaine have the potential to treat ALD, partly via the restoration of transmethylation and transsulfuration pathways of methionine metabolism. However, clinical trials conducted thus far on the effectiveness of SAM for the treatment of ALD are not conclusive. These studies neither support nor refute the use of SAM for the treatment of patients with ALD. In addition, clinical trials on the effectiveness of betaine for the treatment of ALD have not been carried out. Therefore, long-term randomized clinical trials are required to evaluate the safety and efficacy of SAM and of betaine for the treatment of ALD. It is important that factors such as dose, duration of treatment, and bioavailability of compounds should be taken into consideration in the design of these studies. Before clinical trials of folate are considered, additional animal studies are required to determine the potential of folate for the treatment of ALD.

The National Institute on Alcohol Abuse and Alcoholism acknowledges the support provided by the Office of Dietary Supplements (ODS) and the National Center for Complementary and Alternative Medicine (NCCAM) and looks forward to continued cooperation and collaboration. The authors’ responsibilities were as follows—VP: prepared the draft of the manuscript based on materials provided by other authors; all authors: contributed to the review and revision of the draft and approved the final version of the manuscript. None of the authors had any personal or financial conflicts of interest.

REFERENCES


Variations in the preproghrelin gene correlate with higher body mass index, fat mass, and body dissatisfaction in young Japanese women1–3

Tetsuya Ando, Yuhei Ichimaru, Fujiko Konjiki, Masayasu Shoji, and Gen Komaki

ABSTRACT
Background: Ghrelin is an endogenous peptide that stimulates growth hormone secretion, enhances appetite, and increases body weight and may play a role in eating disorders.

Objective: The purpose was to determine whether any preproghrelin gene variants are associated with anthropometric measures, circulating ghrelin, lipid concentrations, insulin resistance, or psychological measures relevant to eating disorders in young women.

Design: This cross-sectional study compared outcome measures between preproghrelin genotypes. The participants in the study included 264 Japanese women [university students with a mean (±SD) age of 20.4 ± 0.7] with no history of eating disorders. The main outcomes were responses to the Eating Disorder Inventory-2 (EDI-2), anthropometric measures, measures of depression and anxiety, and fasting blood concentrations of acylated or desacyl ghrelin, lipids, glucose, and insulin.

Results: Two single nucleotide polymorphisms (SNPs) whose minor allele frequencies were >0.05—the Leu72Met (408 C→A) SNP in exon 2 and the 3056 T→C SNP in intron 2—were used for association analysis. The 3056C allele was significantly associated with a higher acylated ghrelin concentration (P = 0.0021), body weight (P = 0.011), body mass index (P = 0.007), fat mass (P = 0.012), waist circumference (P = 0.008), and skinfold thickness (P = 0.011) and a lower HDL-cholesterol concentration (P = 0.02). Interestingly, the 3056C allele was related to elevated scores in the Drive for Thinness–Body Dissatisfaction (DT-BD) subscale of the EDI-2 (P = 0.003).

Conclusion: Our findings suggest that the preproghrelin gene 3056T→C SNP is associated with changes in basal ghrelin concentrations and physical and psychological variables related to eating disorders and obesity. Am J Clin Nutr 2007;86:25–32.

KEY WORDS Eating disorders, ghrelin, body mass index, body dissatisfaction, polymorphisms, obesity, HDL cholesterol, Eating Disorder Inventory-2

INTRODUCTION
Eating disorders are characterized by severe alterations in eating behavior, body shape perception, and body weight regulation. Genetic factors play an important role in the susceptibility to eating disorders such as anorexia nervosa (AN) and bulimia nervosa (BN) (1–3). In addition, psychopathologies relevant to eating disorders, such as the drive for thinness, body dissatisfaction, and body mass index (BMI), have a considerable genetic component (4, 5). Studies of risk factors (6) found that childhood or parental obesity promotes dieting behavior, and these risk factors are more prominent among persons with BN (7). Thus, candidate genes related to appetite control, energy expenditure, and obesity have been studied in search of a predisposition to eating disorders (8).

Ghrelin is an orexigenic peptide ligand that stimulates growth hormone secretion when it binds to the growth hormone secretagogue receptor (9). Ghrelin is primarily produced by neuroendocrine cells in the stomach fundus (9–11) and induces appetite and increases food intake in rodents and humans (12, 13). Ghrelin secretion is up-regulated under conditions of negative energy balance, such as emaciation, and is down-regulated under conditions of positive energy balance, such as obesity (14, 15). The plasma ghrelin concentration rises during fasting and falls quickly after meal (16). Thus, ghrelin plays a role in the long-term as well as the short-term regulation of feeding.

Early studies on the role of ghrelin in eating disorders indicate that underweight AN patients have elevated plasma ghrelin concentrations and that the concentration returns to normal after weight gain (10, 15). A more recent investigation, however, has indicated that the fasting plasma concentration of biologically

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active ghrelin (acylated ghrelin) is not elevated, whereas a degraded form of ghrelin (desacyl ghrelin) is elevated in AN (17). It was also reported that the normal meal-induced decrease in plasma ghrelin concentrations is blunted in BN (18). Thus, impaired ghrelin regulation may play a role in the etiology and pathology of AN and BN.

The gene encoding preproghrelin, the ghrelin precursor, is located on chromosome 3 at 3p26-25 and comprises 4 exons and 3 introns (19, 20). Three nonsynonymous single nucleotide polymorphisms (SNPs) in the preproghrelin gene have been reported: Arg51Gln SNP is associated with lower ghrelin and insulin-like growth factor I concentrations (21, 22). Leu72Met SNP has been linked to obesity-related phenotypes, but the findings are controversial (19, 22–25). Leu90Gln SNP also has been linked to obesity (26). Recently, we found that the 3056 T→C SNP in intron 2 and the Leu72Met SNP of the preproghrelin gene were significantly associated with purging-type BN (27).

The purpose of the present study was to determine whether any preproghrelin gene polymorphisms are associated with anthropometric, biochemical and psychological measures relevant to eating disorders or obesity in non-clinical young women who are at the ages vulnerable to eating disorders.

SUBJECTS AND METHODS

Subjects

Unrelated woman Japanese volunteers were recruited among university students after excluding those who reported a history of eating disorders or any other psychiatric, digestive, endocrine, or metabolic diseases. We thus obtained data from 264 non-clinical woman subjects. Their mean (±SD) age and current BMI (in kg/m²) were 20.4 ± 0.7 y (range: 19–23 y) and 20.6 ± 2.3 (range: 16.2–33.1), respectively (Table 1).

The menstrual phase of each subject on the day of blood sampling was specified based on the date of the last menses and the subject’s usual menstrual cycle. Seventy subjects were estimated to be in the follicular phase, 94 were in the luteal phase, and 39 were near ovulation. The phases of 61 subjects were not specified because of an irregularity of the cycle or incomplete information.

The ethics committees of the National Center of Neurology and Psychiatry and the Tokyo Kasei University approved the investigation. All subjects gave their written informed consent before participation in the study. Parental consent was obtained for subjects aged <20 y.

Anthropometric measurements

BMI was calculated from measurements of height and weight. Fat mass and lean mass were determined by bioelectrical impedance analysis with a TBF-410 Body Composition Analyzer (TANITA, Tokyo, Japan) according to the manufacturer’s internal algorithm. Waist and hip circumferences were measured by trained personnel using a tape measure, and the waist-to-hip ratio was then calculated. Triceps and subscapular skinfold thicknesses were measured by using a skinfold caliper, and the skinfold thicknesses were summed. Each subject was asked for her lowest and highest weights after she had reached adult height, and minimum and maximum BMI values were calculated.

### TABLE 1

General data and characteristics of the subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Healthy young women (n = 264)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>20.4 ± 0.7 (19–23)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>159.2 ± 5.4 (147.2–176.8)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>52.3 ± 6.7 (39.8–82.3)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.6 ± 2.3 (16.2–33.1)</td>
</tr>
<tr>
<td>Underweight (n = 36)</td>
<td>17.7 ± 0.6 (16.2–18.4)</td>
</tr>
<tr>
<td>Healthy weight (n = 214)</td>
<td>20.7 ± 1.5 (18.5–24.9)</td>
</tr>
<tr>
<td>Overweight (n = 12)</td>
<td>26.4 ± 1.3 (25.1–29.4)</td>
</tr>
<tr>
<td>Obese (n = 2)</td>
<td>31.7 ± 2.1 (30.3–32.1)</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>13.2 ± 4.3 (6.3–39.1)</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>39.1 ± 3.4 (31.8–49.0)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>65.0 ± 5.1 (54–87)</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.71 ± 0.04 (0.64–0.85)</td>
</tr>
<tr>
<td>Sum of skinfold thicknesses (mm)</td>
<td>31.1 ± 12.1 (11–75)</td>
</tr>
<tr>
<td>Maximum BMI (kg/m²)</td>
<td>21.8 ± 2.3 (17.0–33.1)</td>
</tr>
<tr>
<td>Minimum BMI (kg/m²)</td>
<td>19.4 ± 1.9 (15.2–25.7)</td>
</tr>
<tr>
<td>Acylated ghrelin (fmol/mL)</td>
<td>20.3 ± 9.8 (4.2–62.6)</td>
</tr>
<tr>
<td>Desacyl ghrelin (fmol/mL)</td>
<td>148.5 ± 106.4 (32.1–740.0)</td>
</tr>
<tr>
<td>Acylated/desacyl ghrelin</td>
<td>0.16 ± 0.09 (0.008–0.58)</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>181.1 ± 28.6 (115–305)</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>70.3 ± 13.1 (31–110)</td>
</tr>
<tr>
<td>Triacylglycerol (mg/dL)</td>
<td>62.2 ± 24.6 (26–173)</td>
</tr>
<tr>
<td>Free fatty acids (mEq/L)</td>
<td>0.68 ± 0.31 (0.09–1.95)</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>92.7 ± 7.1 (54–120)</td>
</tr>
<tr>
<td>Insulin (µIU/L)</td>
<td>7.09 ± 3.43 (0.96–31.1)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.64 ± 0.84 (0.21–7.67)</td>
</tr>
</tbody>
</table>

1 All values are ± SD; range in parentheses. HOMA-IR, homeostasis model assessment of insulin resistance.
2 Underweight, BMI <18.5; healthy weight, 18.5 ≤ BMI < 25, overweight, 25 ≤ BMI <30; obese, BMI ≥ 30.
3 Minimum and maximum values were calculated on the basis of self-reported lowest and highest weights after reaching adult height.

Blood sampling

Blood was obtained by venipuncture between 0900 and 1100 after an overnight fast of >12 h. Blood for the ghrelin assay was collected into a tube containing 500 KIU aprotinin and 1.25 mg sodium EDTA/mL whole blood, chilled immediately on ice, and centrifuged (1500 × g, 15 min at 4 °C) within 30 min after collection. The plasma was collected, acidified with 1/10 volume of 1 mol HCl/L (28), and then stored at −80 °C until assayed. The blood cell component was stored separately for genetic analysis. The samples for the serum lipids assay and the plasma glucose assay were collected in separate tubes, prepared by standard methods, and stored at −80 °C.

Fasting plasma acylated and desacyl ghrelin measurements

The fasting concentrations of the intact acylated form of ghrelin (acylated ghrelin) and of the degraded desacyl form of ghrelin (desacyl ghrelin) were measured by using 2 commercially available enzyme-linked immunosorbent assay (ELISA) kits, an Active Ghrelin ELISA Kit and a Desacyl-Ghrelin ELISA Kit, respectively, according to the manufacturer’s protocol (Mitsubishi Kagaku Iatron Inc, Tokyo, Japan) (29, 30). The minimal detection limits of acylated and desacyl ghrelin in this assay system were 2.5 and 12.5 fmol/mL, respectively. The intra- and interassay CVs were 4.3% and 3.5%, respectively, for acylated ghrelin and 3.5% and 6.7%, respectively, for desacyl ghrelin.
Fasting blood biochemical measurements

Fasting concentrations of serum total cholesterol, HDL-cholesterol, triacylglycerol, free fatty acids, and plasma glucose and insulin were measured by using standard enzymatic methods with an automated analyzer (SRL, Ltd, Tachikawa, Tokyo, Japan). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting plasma insulin (µU/L) × fasting plasma glucose (mmol/L)/22.5.

SNP selection

We selected 3 nonsynonymous SNPs in the coding region of the preproghrelin gene (OMIM: 605353): in exon 2, the SNPs Arg51Gln (346 G→A) (Celera database ID = hCV25607739) and Leu72Met (408 C→A) (NCBI dbSNP database ID = rs696217); in exon 3, the SNP Leu90Gln (3412 T→A) (rs4864877). We also selected 3 SNPs in the noncoding region of the gene that are reported to be polymorphic in the Japanese population according to the JSNP database (31) or the international HapMap project (32); 3056 T→C (rs2075356) and 3083 A→G (rs35682) in intron 2 and 3615 A→C (rs35683) in intron 3 (Internet: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = SNP).

Genotyping

Genomic DNA was extracted from peripheral blood by using a standard procedure. The genotypings of the Arg51Gln, Leu72Met, 3056 T→C, 3083 A→G, Leu90Gln, and 3615 A→C polymorphisms were performed by using TaqMan SNP Genotyping Assays with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) as described by the manufacturer.

Interviews and questionnaires

A clinical history was taken from each participant by a physician with expertise in eating disorders. Participants who had a history of eating disorders or other psychiatric, digestive, endocrine, or metabolic diseases were eliminated from the study. Each participant was administered a questionnaire on the course of body weight change as well as the Japanese version of the Eating Disorder Inventory-2 (EDI-2) (33), the Beck Depression Inventory-2 (BDI-2) (34), and the State-Trait Anxiety Inventory (STAI) (35). Depression and anxiety are symptoms often accompanied with eating disorders (6, 7).

EDI-2 is a self-rating scale that assesses the multidimensional symptomatology commonly associated with AN and BN (33). A Japanese version of this instrument, which has been translated and validated (M Shoji, personal communication, 2006), consists of 6 factors resulting in a corresponding 6 subscales named Bulimia, Drive for Thinness–Body Dissatisfaction (DT-BD), Interceptive Awareness, Impulse Regulation, Ineffectiveness, and Interpersonal Difficulty. In the original English version of EDI-2, drive for thinness and body dissatisfaction are separate subscales. All the factors indicated satisfactory internal-consistency reliability, with Cronbach’s α coefficients between 0.70 and 0.89. The EDI-2 measurement uses a 6-point Likert scale (5 = always, 0 = never).

Statistical analysis

The chi-square test was used to determine whether the observed genotype frequencies deviated from Hardy-Weinberg equilibrium. Linkage disequilibrium (LD) among the different markers was analyzed with Haploview software (36). For the cross-sectional analysis, all anthropometric variables were compared by one-factor analysis of variance (ANOVA), and all biochemical and psychological variables were compared after adjustment for BMI by analysis of covariance. Values for acylated ghrelin, desacyl ghrelin, the ratio of acylated ghrelin to desacyl ghrelin, insulin, and HOMA-IR were analyzed after logarithmic transformation because of their skewed distribution. A Fisher’s exact test was used to compare frequencies of underweight, normal weight, overweight, and obesity between groups. Correlations between variables were assessed by using Spearman’s correlation coefficients. A P value <0.05 in the 2-tailed test was considered significant. The statistical software was the SAS System for WINDOWS (version 8; SAS Institute Japan, Tokyo, Japan).

RESULTS

General measurements and correlation among variables

The demographic, anthropometric, and metabolic variables are shown in Table 1. There was a good correlation between acylated and desacyl ghrelin concentrations (r = 0.35, P < 0.0001). Correlations of acylated ghrelin, desacyl ghrelin, and the ratio of acylated to desacyl ghrelin with anthropometric and blood biochemical measures are shown in Table 2. Plasma acylated ghrelin concentrations correlated negatively and modestly with BMI, waist circumference, and fat mass, but not with lean mass, waist-to-hip ratio, or skinfold thickness. The acylated ghrelin concentration also correlated negatively with insulin and HOMA-IR. The desacyl ghrelin concentration showed negative and weak correlations with BMI, fat mass, waist circumference, waist-to-hip ratio, and sum of skinfold thicknesses. Desacyl ghrelin correlated positively and weakly with serum free fatty acid and negatively with insulin and HOMA-IR. The ratio of acylated to desacyl ghrelin showed a weak positive correlation with skinfold thickness and a negative correlation with free fatty acids.

Of the psychometric measures, the Bulimia subscale scores of the EDI-2 showed positive correlations with BMI, fat mass, lean mass, skinfold thickness, waist circumference, and waist-to-hip ratio and a weak negative correlation with acylated ghrelin concentration (Table 3). The DT-BD scores correlated positively with BMI, fat mass, lean mass, waist circumference, waist-to-hip ratio, and skinfold thickness and negatively with HDL-cholesterol concentrations (Table 3). DT-BD did not correlate with the plasma acylated ghrelin concentration. The other EDI-2 subscale scores—BDI-2 and STAI scores—did not correlate with any anthropometric or blood measures (data not shown).

Estimation of genotype and allele frequencies and LD among SNPs in the sample set

Four SNPs (Arg51Gln, 3083 A→G; Leu90Gln, and 3615 A→C) that had a low (<0.05) minor allele frequency in our samples were excluded from further association analysis to avoid a genotype that occurs at a very low frequency (data not shown). MAFs for the remaining 2 SNPs, Leu72Met and 3056 T→C were 0.168 and 0.267, respectively. The 2 SNPs were in strong LD (pairwise D’ = 0.827, r2 = 0.368). Genotype frequencies [n (%)] of the Leu72Met SNPs were as follows: Met/Met, 10 (3.8); Leu/Met, 69 (26.1); and Leu/Leu, 185 (70.1). The frequencies of
the 3056 T→C genotypes were as follows: C/C, 23 (8.7); T/C, 95 (36.0); and T/T, 146 (55.3). The genotype distributions of both the Leu72Met and 3056 T→C SNPs followed Hardy-Weinberg equilibrium (P > 0.05, chi-square test).

Comparison of anthropometric measures between preproghrelin genotypes

Comparisons of anthropometric data among the preproghrelin genotypes are shown in Table 4. The Leu72Met SNP was not related to any differences in the anthropometric values. In contrast, the subjects with the 3056C allele (C3056C and T3056C genotypes) had a significantly higher mean current body weight (one-factor ANOVA), BMI, fat mass, waist circumference, sum of skinfold thicknesses, and self-reported past minimum and maximum BMIs than did the T3056T genotype. There was a significant difference in the frequencies of subgroups based on the BMI between 3056 T→C genotypes (Fisher’s exact test). There were more overweight or obese and less underweight subjects in the 3056C allele group than in the T3056T genotype.

Comparison of fasting concentrations of acylated and desacyl ghrelin, lipids, and glucose and insulin resistance between preproghrelin genotypes

 Plasma concentrations of acylated ghrelin, desacyl ghrelin, and acylated-desacyl ratios; serum lipids, glucose, and insulin, and the HOMA-IR are shown in Table 5. Subjects with the 3056C allele had significantly higher acylated ghrelin concentrations (P = 0.0021, P = 0.020, and P = 0.019 after adjustment for BMI, insulin and, HOMA-IR, respectively; ANCOVA) and lower HDL-cholesterol concentrations than did those with the T3056T genotype. The 72Met carriers (Met72Met and Leu72Met genotypes) also had higher acylated ghrelin concentrations than

<table>
<thead>
<tr>
<th>Acylated ghrelin</th>
<th>Desacyl ghrelin</th>
<th>Acylated/desacyl ghrelin</th>
</tr>
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<tbody>
<tr>
<td>r</td>
<td>P</td>
<td>r</td>
</tr>
<tr>
<td>BMI</td>
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<tr>
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<td>Lean mass</td>
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<td>Waist circumference</td>
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<td>Waist-to-hip ratio</td>
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<td>Sum of skinfold thicknesses</td>
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<tr>
<td>Total cholesterol</td>
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<tr>
<td>HDL cholesterol</td>
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<tr>
<td>Triacylglycerol</td>
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<td>Free fatty acids</td>
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<td>Glucose</td>
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<td>0.48</td>
</tr>
<tr>
<td>Insulin</td>
<td>−0.16</td>
<td>0.008</td>
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<tr>
<td>HOMA-IR</td>
<td>−0.17</td>
<td>0.006</td>
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</table>

<table>
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<tr>
<th>Table 2</th>
<th>Spearman’s correlation coefficients for the relation of acylated ghrelin, desacyl ghrelin, and the ratio of acylated to desacyl ghrelin with anthropometric and blood measures¹</th>
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<tr>
<td></td>
<td>Acylated ghrelin</td>
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<tr>
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<td>Sum of skinfold thicknesses</td>
<td>−0.06</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.03</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.04</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>−0.07</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>−0.10</td>
</tr>
<tr>
<td>Glucose</td>
<td>−0.04</td>
</tr>
<tr>
<td>Insulin</td>
<td>−0.16</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>−0.17</td>
</tr>
</tbody>
</table>

¹ HOMA-IR, homeostasis model assessment of insulin resistance. P values ≤ 0.05 indicate significance.

Table 3

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Spearman’s correlation coefficients for the relation of bulimia with the drive for thinness–body dissatisfaction scores on the Eating Disorder Inventory-2 (EDI-2) with anthropometric and blood measures¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bulimia</td>
</tr>
<tr>
<td></td>
<td>r</td>
</tr>
<tr>
<td>BMI</td>
<td>0.38</td>
</tr>
<tr>
<td>Fat mass</td>
<td>0.39</td>
</tr>
<tr>
<td>Lean mass</td>
<td>0.25</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>0.39</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.25</td>
</tr>
<tr>
<td>Sum of skinfold thicknesses</td>
<td>0.32</td>
</tr>
<tr>
<td>Acylated ghrelin</td>
<td>−0.14</td>
</tr>
<tr>
<td>Desacyl ghrelin</td>
<td>−0.04</td>
</tr>
<tr>
<td>Acylated/desacyl ghrelin</td>
<td>−0.08</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.06</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>−0.11</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>0.06</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>−0.12</td>
</tr>
<tr>
<td>Glucose</td>
<td>−0.10</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.08</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.07</td>
</tr>
</tbody>
</table>

¹ HOMA-IR, homeostasis model assessment of insulin resistance. P values ≤ 0.05 indicate significance.
TABLE 4
Comparisons of age and anthropometric measures between the genotypes of the preproghrelin single nucleotide polymorphisms

<table>
<thead>
<tr>
<th>Variable</th>
<th>Leu72Met (408 C→A)</th>
<th>3056 T→C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA + CA (n = 79)</td>
<td>CC (n = 185)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>20.5 ± 0.7</td>
<td>20.3 ± 0.7</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>53.5 ± 7.1</td>
<td>51.8 ± 6.5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.9 ± 2.6</td>
<td>20.5 ± 2.2</td>
</tr>
<tr>
<td>Underweight [n (%)]</td>
<td>8 (10.1)</td>
<td>28 (15.1)</td>
</tr>
<tr>
<td>Healthy weight [n (%)]</td>
<td>64 (81.0)</td>
<td>150 (81.1)</td>
</tr>
<tr>
<td>Overweight [n (%)]</td>
<td>6 (7.6)</td>
<td>6 (3.2)</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>13.8 ± 4.8</td>
<td>12.9 ± 4.1</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>65.5 ± 5.1</td>
<td>64.8 ± 5.1</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.71 ± 0.03</td>
<td>0.71 ± 0.04</td>
</tr>
<tr>
<td>Sum of skinfold thicknesses (mm)</td>
<td>32.4 ± 13.2</td>
<td>30.6 ± 11.6</td>
</tr>
<tr>
<td>Maximum BMI (kg/m²)</td>
<td>22.3 ± 2.6</td>
<td>21.6 ± 2.1</td>
</tr>
<tr>
<td>Minimum BMI (kg/m²)</td>
<td>19.7 ± 1.9</td>
<td>19.3 ± 1.9</td>
</tr>
</tbody>
</table>

* P values refer to group differences in means by one-factor ANOVA.
² ± SD (all such values).
³ P values ≤ 0.05 indicate significance.
⁴ P value refers to group differences in frequencies by Fisher’s exact test.
5 Calculated on the basis of self-reported lowest and highest weights after adult height was reached. Underweight, BMI < 18.5; healthy weight, 18.5 ≤ BMI < 25; overweight, 25 ≤ BMI < 30; obese, BMI ≥ 30.

Table 5
Comparisons of blood biochemical measures between the genotypes of the preproghrelin single nucleotide polymorphisms

<table>
<thead>
<tr>
<th>Variable</th>
<th>Leu72Met (408 C→A)</th>
<th>3056 T→C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA + CA (n = 79)</td>
<td>CC (n = 185)</td>
</tr>
<tr>
<td>Acylated ghrelin (fmol/mL)</td>
<td>21.9 ± 6.4</td>
<td>19.7 ± 9.9</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Underweight</td>
<td>29.0 ± 12.⁴</td>
<td>19.2 ± 6.9</td>
</tr>
<tr>
<td>Healthy weight</td>
<td>21.6 ± 9.1</td>
<td>20.2 ± 10.3</td>
</tr>
<tr>
<td>Overweight or obese</td>
<td>16.2 ± 7.5</td>
<td>9.6 ± 6.3</td>
</tr>
<tr>
<td>Desacylated ghrelin (fmol/mL)</td>
<td>147.3 ± 85.2</td>
<td>149.0 ± 114.5</td>
</tr>
<tr>
<td>Acylated/desacylated ghrelin</td>
<td>0.172 ± 0.091</td>
<td>0.160 ± 0.084</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>180.5 ± 26.2</td>
<td>181.3 ± 29.7</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>69.1 ± 11.7</td>
<td>70.8 ± 13.6</td>
</tr>
<tr>
<td>Triacylglycerol (mg/dL)</td>
<td>60.5 ± 22.7</td>
<td>63.0 ± 25.4</td>
</tr>
<tr>
<td>Free fatty acids (mEq/L)</td>
<td>0.71 ± 0.35</td>
<td>0.66 ± 0.29</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>92.9 ± 5.8</td>
<td>92.7 ± 7.6</td>
</tr>
<tr>
<td>Insulin (µIU/L)</td>
<td>7.46 ± 3.93</td>
<td>6.93 ± 3.20</td>
</tr>
<tr>
<td>HOMA-IR (³)</td>
<td>1.72 ± 0.97</td>
<td>1.61 ± 0.79</td>
</tr>
</tbody>
</table>

¹ All values are ± SD. HOMA-IR, homeostasis model assessment of insulin resistance.
² P values refer to group differences in means after adjustment for BMI by ANCOVA.
³ Values were analyzed after logarithmic transformation because of skewed distribution.
⁴ P values ≤ 0.05 indicate significance.
⁵ P = 0.047 and 0.035 after adjustment for insulin and HOMA-IR, respectively.
⁶ P = 0.020 and 0.019 after adjustment for insulin and HOMA-IR, respectively.
⁷ Underweight, BMI < 18.5; healthy weight, 18.5 ≤ BMI < 25; overweight, 25 ≤ BMI < 30; obese, BMI ≥ 30.
The findings have been controversial. The role of the common Leu72Met SNP has been studied with acylated ghrelin concentration, but to a lesser extent. A study by Ando et al. showed that individuals with the minor 3056 C→T SNP genotypes were associated with higher obesity-related anthropometric measures, elevated fasting acylated ghrelin concentrations, and lower serum HDL-cholesterol concentrations. Moreover, the subjects with the 3056 C→T SNP allele had higher scores on the DT-BD subscale than did those with the Leu72Met genotype (P = 0.018, ANOVA), but the association diminished after adjustment for BMI (P = 0.066). The 3056C allele carriers of the 3056 T→C SNP also had significantly higher DT-BD scores, even after adjustment for BMI. The remaining EDI-2 subscale, depression scale (BDI-2), state anxiety scale (STAI-state), and trait anxiety scale (STAI-trait) did not differ significantly between the genotypes.

### Discussion

In this study, we provided evidence that the minor 3056C allele of the 3056 T→C SNP in intron 2 of the preproghrelin gene is associated with higher obesity-related anthropometric measures, elevated fasting acylated ghrelin concentrations, and lower serum HDL-cholesterol concentrations. Moreover, the subjects with the 3056 C→T SNP allele had higher scores on the DT-BD subscale of the EDI-2, which is one of the psychopathologies characteristic of eating disorders. The Leu72Met SNP in exon 2, which is in strong LD with the 3056 T→C SNP, showed an association with acylated ghrelin concentration, but to a lesser extent.

We previously found that the 3056 T→C and the Leu72Met SNPs, and the haplotype formed by the 2 SNPs, are associated with the susceptibility to purging-type BN in Japanese (27). Our current findings are generally consistent with these findings in BN patients, because premorbid obesity and body dissatisfaction are widely recognized as important risk factors for BN (37).

The role of the common Leu72Met SNP has been studied intensively with regard to obesity-related phenotypes, but the findings have been controversial. The 72Met allele has been associated with an earlier onset of obesity (19, 25) and with a positive family history for obesity (24). Obese children carrying the 72Met allele have higher BMIs than those carrying only the 72Leu allele (23). Studies with normal-weight, healthy individuals, however, have yielded opposite results. The Met72Met genotype was associated with lower BMI, fat mass, and abdominal visceral fat in white individuals, and the 72Met allele was associated with lower fat mass and higher insulin-like growth factor 1 concentrations in individuals of African descent (22).

In the current study, the 3056C allele was associated consistently with most of the anthropometric variables related to obesity. Thus, the 3056C allele is more likely to be the actual risk-conferring allele than is the 72Met allele, and this might be the reason for the controversial findings concerning the Leu72Met SNP and obesity-related phenotypes.

We showed for the first time that the 3056 T>C SNP is related to fasting acylated ghrelin concentration. When the subjects were subdivided by BMI, the difference in acylated ghrelin was evident in the underweight subjects. On the other hand, we found no difference in desacyl ghrelin or in the ratio of acylated to desacyl between the preproghrelin genotypes. Previous studies, in which only total ghrelin was measured, found no changes in basal ghrelin concentrations because of the Leu72Met SNP (22, 24). N-Octanoylation of the Ser-3 hydroxy group is thought to be essential for the biological activity of ghrelin (acylated ghrelin) (38). The acylated ghrelin is quite unstable and is rapidly degraded to the des-octanoyl form (desacyl ghrelin) (39). Desacyl ghrelin, however, was recently reported to have some functions, such as the stimulation of food intake (40) and the inhibition of isoproterenol-induced lipolysis (41) in rodents.

In our sample, both acylated and desacyl ghrelin concentrations correlated negatively with BMI, insulin, and insulin resistance in agreement with previous studies (14, 15, 42). However, the difference in acylated ghrelin concentrations due to the 3056C allele remained significant even after adjustment for BMI, insulin, and HOMA-IR, which suggests that the effect of the 3056C allele was not secondary to changes in BMI or insulin resistance. HDL cholesterol correlated negatively with BMI, but not with acylated or desacyl ghrelin, and its association with the 3056 C→T SNP allele was at least partially dependent on changes in body size and fat measures.

The physiologic significance for the 2.8-fmol/mL difference in plasma acylated ghrelin concentrations is unclear. It has been.

### Table 6

Scores on the Eating Disorder Inventory-2 (EDI-2), Beck Depression Inventory-2 (BDI-2), and State-Trait Anxiety Inventory (STAI) by preproghrelin single nucleotide polymorphisms

<table>
<thead>
<tr>
<th></th>
<th>Leu72Met (408 C→A)</th>
<th>3056 T→C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA + CA (n = 79)</td>
<td>CC (n = 185)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulimia</td>
<td>20.1 ± 9.8</td>
<td>18.5 ± 9.4</td>
</tr>
<tr>
<td>Drive for Thinness–Body Dissatisfaction</td>
<td>26.1 ± 7.7</td>
<td>23.4 ± 8.1</td>
</tr>
<tr>
<td>Interoceptive Awareness</td>
<td>8.8 ± 5.9</td>
<td>9.2 ± 5.0</td>
</tr>
<tr>
<td>Impulse Regulation</td>
<td>9.3 ± 5.1</td>
<td>8.5 ± 4.4</td>
</tr>
<tr>
<td>Ineffectiveness</td>
<td>12.4 ± 5.3</td>
<td>11.6 ± 4.9</td>
</tr>
<tr>
<td>Interpersonal Difficulty</td>
<td>9.3 ± 3.8</td>
<td>8.8 ± 4.0</td>
</tr>
<tr>
<td>BDI-2</td>
<td>12.4 ± 8.0</td>
<td>11.5 ± 7.9</td>
</tr>
<tr>
<td>STAI-state</td>
<td>44.0 ± 12.2</td>
<td>42.7 ± 10.4</td>
</tr>
<tr>
<td>STAI-trait</td>
<td>47.9 ± 11.5</td>
<td>46.6 ± 11.0</td>
</tr>
</tbody>
</table>

1 All values are x ± SD.
2 P values refer to group differences in means after adjustment for BMI by ANCOVA.
3 P values ≤ 0.05 indicate significance.
indicated that the gastric vagal afferent, rather than blood circulation, is the major pathway conveying ghrelin’s signals for starvation and GH secretion to the brain (43). If so, the plasma concentration is not a direct index of actual ghrelin action but rather an indicator of the status of ghrelin secretion and metabolism.

The Leu72Met SNP of the preproghrelin gene is outside the region encoding the mature ghrelin product, and its functional significance remains unclear (19). The molecular mechanisms of the effect of the 3056 T→C SNP has not yet been studied; however, an intronic SNP possibly affects gene expression and mRNA stability (44).

The EDI-2 DT-BD scores were elevated in subjects with the 3056C allele. The DT-BD factor consists of items such as fear of fatness, desire for thinness, and dissatisfaction with one’s own body size and shape. Considerable heritability for the BD (52~59%) and DT (44~51%) scores and also relatively high heritability for perfectionism (43%) and present BMI (64%) have been reported in twin studies (4, 5). Our current findings suggest that the preproghrelin gene may contribute to the heritability of the BD and DT scores and BMI.

The nature of the direction of causality among the ghrelin concentrations, anthropometric measures, and psychological variables is unclear. We speculate that a slight, but long-lasting, increment in ghrelin action due to the 3056C allele in the preproghrelin gene results in a larger BMI and higher body fat by enhancing appetite, growth hormone release, and fat deposition (9, 12, 13). Our current data and previous studies showed positive correlations among DT and BD scores and body size measures (5). In addition to the elevated body size and adiposity, physical factors such as weight fluctuation and poor physical form and psychosocial factors such as severe life stress, negative affection, and perceived peer pressure to be thin are risk factors for body dissatisfaction (45, 46). Although the ghrelin/growth hormone secretagogue receptor system is involved in the modulation of the hypothalamic-pituitary-adrenal axis response to stress in humans (47), the relation between ghrelin and these psychosocial risk factors is not known.

We recruited the women for the current study from healthy university students in the Tokyo metropolitan area who were of similar age (~20 y) and who presumably shared similar social values and lifestyles. The subjects were relatively free from long-term effects of lifestyle habits and secondary changes due to diseases. These characteristics of the sample subjects, in theory, allow for better detection of small genetic effects on anthropometric and blood-composition variables. Although the expression of ghrelin may be affected by estrogen or menstrual phase (48), we found no differences in ghrelin concentrations between the menstrual phases nor in the frequencies of the menstrual phases between the preproghrelin genotypes. Because the estrogen concentration fluctuates within each menstrual phase, adjustment for blood estrogen concentrations will be needed to exclude the possible confounding effect of estrogen on basal ghrelin concentrations. Studies in a male population will be necessary to validate the current findings and to test possible sex differences in the effect of preproghrelin variants.

The mean BMI of Japanese women aged 20–24 was 20.39 ± 2.70 in 1996–2000 (49). Therefore, the constitution of our subjects (BMI: 20.4 ± 0.7) was average for the Japanese. Nevertheless, the desire of young women for thinness is greater than other age groups (50). The 3056C allele carriers were relatively larger and more dissatisfied with their body than were noncarriers. Further studies are needed to determine whether 3056C allele carriers are more inclined to develop eating disorders or obesity in the future than are noncarriers.

In conclusion, the 3056C allele was associated with higher body size and fat measures, basal ghrelin concentrations, and drive for thinness and body dissatisfaction in young women. Our current findings should be confirmed by further studies and in other populations. The molecular mechanisms of the effect of the 3056C allele on these variables, which are related to eating disorders and obesity, remain to be elucidated.

We thank Naoko Tachikawa, Shizuka Fukadai, and Yuko Kamiya for their technical assistance with the laboratory analyses.

The authors’ responsibilities were as follows—TA: conceived of and designed the study, directed the laboratory analyses, performed the statistical analysis, and wrote the manuscript; YI and FK: participated in designing the study and helped recruit the participants, conduct the anthropometric measurements, collect the blood samples; MS: performed the psychological assessments; GK: supervised the study and helped design the study and write the manuscript. None of the authors had any financial or personal conflicts of interest.

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17. Purnell JQ, Weigle DS, Breen P, Cummings DE. Ghrelin levels correlate with insulin levels, insulin resistance, and high-density lipoprotein cholesterol, but not with gender, menopausal status, or cortisol levels in humans. J Clin Endocrinol Metab 2003;88:5747–52.


Relation of body mass index and waist-to-height ratio to cardiovascular disease risk factors in children and adolescents: the Bogalusa Heart Study1–4

David S Freedman, Henry S Kahn, Zuguo Mei, Laurence M Grummer-Strawn, William H Dietz, Sathanur R Srinivasan, and Gerald S Berenson

ABSTRACT

Background: Several investigators have concluded that the waist-to-height ratio is more strongly associated with cardiovascular disease risk factors than is the body mass index (BMI; in kg/m²).

Objectives: We examined the relation of the BMI-for-age z score and waist-to-height ratio to risk factors (lipids, fasting insulin, and blood pressures). We also compared the abilities of these 2 indexes to identify children with adverse risk factors.

Design: Children aged 5–17 y (n = 2498) in the Bogalusa Heart Study were evaluated.

Results: As assessed by the ability of the 2 indexes to 1) account for the variability in each risk factor and 2) correctly identify children with adverse values, the predictive abilities of the BMI-for-age z score and waist-to-height ratio were similar. Waist-to-height ratio was slightly better (0.01–0.02 higher R² values, P < 0.05) in predicting concentrations of total-to-HDL cholesterol ratio and LDL cholesterol, but BMI was slightly better in identifying children with high systolic blood pressure (0.03 higher R², P < 0.05) in predicting measures of fasting insulin and systolic and diastolic blood pressures. On the basis of an overall index of the 6 risk factors, no difference was observed in the predictive abilities of BMI-for-age and waist-to-height ratio, with areas under the curves of 0.85 and 0.86 (P = 0.30) and multiple R² values of 0.320 and 0.318 (P = 0.79). This similarity likely results from the high intercorrelation (R² = 0.78) between the 2 indexes.

Conclusions: BMI-for-age and waist-to-height ratio do not differ in their abilities to identify children with adverse risk factors. Although waist-to-height ratio may be preferred because of its simplicity, additional longitudinal data are needed to examine its relation to disease.

KEY WORDS BMI, body mass index, waist, height, waist-to-height ratio, children, lipids, blood pressure, insulin

INTRODUCTION

Vague (1) was the first to observe that women with android obesity had a high prevalence of diabetes and atherosclerosis. Subsequent studies have shown that abdominal obesity, as measured by the waist circumference or related indexes such as the waist-to-hip ratio, is associated with the subsequent development of type 2 diabetes (2–5) and ischemic heart disease (6–8), as well as with risk factors for cardiovascular disease (CVD) (9). Furthermore, despite the relatively low amount of intraabdominal fat among children (10), several indexes of abdominal obesity are associated with CVD risk factors among children and adolescents (11–16).

The waist-to-height ratio was first used in the Framingham Study (17), and several studies of children (13–15) and adults (18, 19) have concluded that this ratio is more strongly associated with CVD risk factors than is the body mass index (BMI; in kg/m²). In addition, waist-to-height ratio may be simpler to use. For example, because waist-to-height ratio is only weakly associated with age, measures among children do not have to be expressed relative to their sex and age peers [by using z scores (20)] as do measures of BMI. In addition, the same cutoff (eg, 0.5) could possibly be used to identify adverse measures of waist-to-height ratio among both children and adults (21, 22), which would simplify the expression of obesity-related disease risk. However, relatively few studies have examined the relation of waist-to-height ratio to CVD risk factors, and it is important to examine these associations in other data.

The current study compares the relation of BMI and waist-to-height ratio to measures of lipids, fasting insulin, and blood pressure among 5–17-y-olds (n = 2498) in the Bogalusa Heart Study. In addition, we examine the abilities of these 2 indexes to correctly identify children with adverse risk factors.

SUBJECTS AND METHODS

Study population

The Bogalusa (Louisiana) Heart Study is a community-based (Ward 4 of Washington Parish) study of CVD risk factors in early life (23). Seven cross-sectional examinations of schoolchildren were conducted since 1973, and the current analyses are based on

1 From the Divisions of Nutrition and Physical Activity (DSF, ZM, LMG-S, and WHD) and Diabetes Translation (HSK), Centers for Disease Control and Prevention, Atlanta, GA, and the Tulane Center for Cardiovascular Health, Tulane University School of Public Health and Tropical Medicine, New Orleans, LA (SRS and GSB).
2 The findings and conclusions in this report are those of the authors and not necessarily those of the CDC.
3 Supported by grant no. AG-16592 from the National Institutes of Aging.
4 Address reprint requests to DS Freedman, CDC K-26, 4770 Buford Highway, Atlanta GA 30341. E-mail: dfreedman@cdc.gov.
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the 1993–1994 examination. Written informed consent was obtained from all parents, and study protocols were approved by human subjects review committees at the Tulane University School of Public Health and Tropical Medicine.

Of the 3135 children and adolescents (aged 5–17 y) examined, we excluded 9 girls who reported being pregnant, 7 children who were not white or black, 30 children who reported taking insulin (or were unsure), 13 children for whom we did not have a systolic (SBP) or diastolic (DBP) blood pressure measurement, and 14 children for whom information on measurements of waist, height, or weight was missing; these categories were not mutually exclusive. Of the remaining 3066 children, cholesterol (total, LDL, and HDL) and triacylglycerol determinations were available for 2961. Nonfasting children were excluded from the analyses of triacylglycerol and fasting insulin concentrations, and another 130 children did not have an insulin determination. After these exclusions, sample sizes for the various risk factors are 3066 (for SBP and DBP), 2961 (for LDL and HDL cholesterol), 2624 (for triacylglycerol), and 2494 (for insulin).

Because obesity is associated positively with LDL cholesterol and negatively with HDL cholesterol, we did not examine associations with total cholesterol. However, the ratio of total cholesterol to HDL cholesterol (total:HDL cholesterol) is included in the analyses.

General examinations

Height was measured to the nearest 0.1 cm with the use of an Iowa Height Board, and weight was measured to the nearest 0.1 kg with the use of a balance beam metric scale; BMI was calculated as a measure of relative weight. No adjustments were made for the weight of the gown, underpants, or socks that were worn during the examination.

BMI z scores were calculated from the 2000 Centers for Disease Control and Prevention (CDC) Growth Charts (20, 24) to account for the differences in BMIs by sex and age. These growth charts express the BMIs of children in the current study relative to their sex and age peers in the United States between 1963 and 1980; the calculated z scores are termed “BMI-for-age” in the current analyses. (BMIs among 5-y-olds in the CDC Growth Charts also include data from 1988–1994.) Overweight is defined as a BMI-for-age z score ≥ 1.645 (corresponding to the 95th percentile of normally distributed data) of these growth charts (25, 26). BMI-for-age z scores were used in all analyses in the current study. BMI-for-age percentile scores are used only to classify children into 4 categories in one table that cross-classifies BMI-for-age and waist-to-height ratio.

The waist circumference was measured midway between the rib cage and the superior border of the iliac crest while the child was standing. Three measurements were obtained with a nonstretchable tape, and the mean value was used in the calculation of the waist-to-height ratio. In analyses that compared the abilities of BMI and waist-to-height ratio to correctly identify children with adverse risk factors, we dichotomized waist-to-height ratio at 0.512 (without considering the child’s sex or age) so that the same proportion (17%) of children would be overweight and have a “high” waist-to-height ratio.

On each examination day, a 10% sample of the children was randomly selected to be reexamined 2–3 h later by the same observer. We use these data to compare the reproducibilities of BMI and waist-to-height ratio.

**Risk factors**

Concentrations of serum total cholesterol and triacylglycerols were measured by using enzymatic procedures in a centralized laboratory that met the requirements of the CDC’s Lipid Standardization Program. For LDL- and HDL-cholesterol measurements, we used a combination of heparin-calcium precipitation and agar-agarose gel electrophoresis (27). Plasma insulin measurements were obtained with the use of a radioimmunoassay procedure (Phadebas Insulin Kit; Pharmacia Diagnostics AB, Uppsala, Sweden).

As previously described (23), sitting SBP and DBP in the right arm were measured 6 times by trained observers with a mercury sphygmomanometer (Baumanometer; WA Baum Co Inc, Copiague, NY). The cuff size was based on the length and circumference of the upper arm and was chosen to be as large as possible without having the elbow skin crease obstruct the stethoscope (28).

The distributions of lipid and lipoprotein concentrations in the Bogalusa Study were similar to those in the third National Health and Nutrition Examination (NHANES III) conducted from 1988 to 1994 (29). For example, the 90th percentiles of LDL cholesterol among 12–15-y-old white children (data were not cross-classified by race, sex, or age group) in NHANES III were 122 mg/dL (whites) and 133 mg/dL (blacks); corresponding values in the Bogalusa Study were 127 mg/dL (whites) and 133 mg/dL (blacks). Similarly, the 10th percentiles of HDL cholesterol were 35 mg/dL (boys) and 36 mg/dL (girls) among 12–15-y-olds in NHANES III and were 37 mg/dL among both boys and girls in the Bogalusa Study. However, because of differences in methods of measuring blood pressure (28), recorded measures of blood pressure are ≈5–10 mm Hg lower in the Bogalusa Study than in other studies.

**Measures of adverse risk factors**

Because measures of lipids, insulin, and blood pressures vary substantially by sex and age, we defined “adverse” measures in relation to a child’s sex and age peers in the Bogalusa Study sample. After log-transformation of measures of the risk factors to improve normality, each risk factor was regressed on sex, race, and age. Age was modeled with the use of restricted cubic splines (5 knots (see Statistical analyses) (30), and we allowed for interactions with age (age × BMI and age × waist-to-height ratio) in the prediction of each risk factor. Regression models for SBP and DBP also included height (cubic splines) as a predictor. The standardized residuals (adjusted risk factor measures) from these models represent measures relative to children of the same sex, race, and age. All adjusted risk factors had a mean ± SD value of 0 ± 1.0. With the exception of HDL cholesterol (<10th percentile), adverse risk factor measures were defined as a measure ≥ 90th percentile.

Although the identification of children with adverse risk factors in the current study is based solely on the distribution of risk factors in the Bogalusa Study, the use of cutoffs from NHANES III (29) identified similar children with adverse concentrations of lipids and lipoproteins. For example, all of the 12–15-y-olds (whites and blacks combined; n = 106) in the current study who were classified as having a high LDL-cholesterol concentration (according to the Bogalusa Study cutoffs) also had a concentration >90th percentile (119 mg/dL) in NHANES III. However, 45 (5%) of the 950 children aged 12–15 y who we considered to have
a “normal” LDL-cholesterol concentration were in the >90th percentile in NHANES III. [It should be noted that some estimates of the 90th percentile in NHANES III were considered to be unstable because of the relatively small sample size (29).] Because of differences in methods of measuring blood pressure (28), few children in the Bogalusa Study had a SBP or DBP > 90th percentile of the National High Blood Pressure Education Program (31).

The risk factor sum was used as a summary measure of the 6 risk factors and was derived by combining adjusted measures of triacylglycerols, LDL cholesterol, HDL cholesterol, fasting insulin, SBP, and DBP. Adjusted measures of most risk factors were simply added together, but adjusted measures of HDL cholesterol were subtracted from the total. In addition, because of the high correlation (r = 0.66) between SBP and DBP, these 2 characteristics were first divided by 2. The resulting risk factor sum had a mean ± SD value of 0 ± 2.9 (range: 1–11). Correlations between the risk factor sum and the individual risk factors ranged from r = 0.37 (DBP) to r = 0.73 (triacylglycerols); the association with HDL cholesterol was r = −0.59.

The risk factor sum was highly correlated (r = 0.97) with the first principal component (32) of the 6 risk factors. Furthermore, with the exception of LDL-cholesterol concentrations (r = 0.39), the absolute value of the correlation coefficients with the first principal component ranged from 0.50 (HDL cholesterol) to 0.70 (triacylglycerols). (The second principal component was difficult to interpret because it contrasted measures of DBP, SBP, and HDL cholesterol with measures of triacylglycerols and fasting insulin, and it was not considered further.) Although risk factor summaries can be derived by adding together the number of adverse risk factors (16, 33), our method allows the risk factor sum to be used as a continuous variable.

Statistical analyses

The analyses, which were performed with the use of SAS software (version 9.1; SAS Institute Inc, Cary, NC) and R [version 2.4.1; R Foundation for Statistical Computing, Vienna, Austria (34)], first examined the ability of BMI-for-age and waist-to-height ratio to identify children with adverse measures of each risk factor. We calculated the positive predictive value (the proportion of children with a high BMI or waist-to-height ratio who actually have adverse risk factors) and the sensitivity (the proportion of children with adverse risk factors who have a high BMI or waist-to-height ratio) for each risk factor. Because these values depend on the cutoff used for BMI and waist-to-height ratio, we also examined the receiver operating characteristic curve for each risk factor. These curves are constructed by plotting the sensitivity at each value of BMI-for-age or waist-to-height ratio compared with the corresponding 1-specificity, and the area under the curve (AUC) quantifies the screening performance over all cutoffs. An AUC of 0.5 indicates that the screening test is no better than chance, and 1.0 indicates perfect classification. The statistical significance of the difference (35) in AUCs between BMI and waist-to-height ratio was calculated by using MEDCALC software (version 9.1.0.1; MedCalc Software, Mariakerke, Belgium).

Regression models were also used to quantify the prediction of risk factor measures by both indexes. (The original, unadjusted measures of the risk factors were used as the dependent variable in these models.) These analyses compared the increases in the multiple R² values achieved by adding either BMI-for-age z score or waist-to-height ratio to a model already containing age, sex, and race. Continuous variables were modeled by using restricted cubic splines with 5 knots (30) to allow for nonlinearity, and we allowed for an interaction between each index and age. In contrast to the use of higher-order polynomials, models based on splines do not have peaks and valleys, and the fit in one region does not influence the fit in all other regions of the data.

To assess the differences between the R² values of models that contained either BMI-for-age or waist-to-height ratio, we first calculated predicted risk factor measures from each model. We then examined the statistical significance of the difference in the correlation between the actual risk factor measures and the 2 sets of predicted risk factor measures coefficients (36). We also examined whether the relation of BMI-for-age and waist-to-height ratio to each risk factor was nonlinear.

We then cross-classified categories of BMI-for-age (<50th percentile, 50th–84th percentile, 85th–94th percentile, and ≥95th percentile) and waist-to-height ratio. Cutoffs for the 4 categories of waist-to-height ratio were selected so that the number of children in each category would equal the number in the corresponding BMI-for-age category. We focused on measures of the risk factor sum among children whose BMI-for-age stratum was lower or higher (discordant) than the corresponding waist-to-height ratio stratum. We also show the relation of waist-to-height ratio to BMI-for-age by using loweress to smooth the data (37).

In addition to determining whether differences between BMI-for-age and waist-to-height ratio were statistically significant, we also focused on the magnitudes of the differences in the AUCs and R² values for each risk factor. It should be realized that a small difference between the 2 indexes, which indicates that their predictive abilities are similar, could be statistically significant. However, this small difference would have little practical importance.

RESULTS

Mean measures of various characteristics are shown in Table 1. The mean BMI-for-age z score was 0.46, and 17% of the children were overweight; BMIs did not differ significantly between boys and girls. The mean waist-to-height ratio was 0.458, and measures of waist, height, waist-to-height ratio, and SBP were slightly but significantly higher among boys than among girls. In contrast, girls had slightly but significantly higher concentrations of total cholesterol:HDL cholesterol, triacylglycerols, LDL cholesterol, and fasting insulin than did boys. Measures of the risk factor sum, an overall summary of the 6 age- and sex-adjusted risk factors, ranged from −9 to 11, with a mean value of 0. Age was associated with BMI (r = 0.48), waist circumference (r = 0.59), and height (r = 0.90) but not with BMI-for-age (r = 0.02) or waist-to-height ratio (r = −0.01). Among the 276 children who were reexamined by the same observer, the intraclass correlations between the repeated measurements were 0.997 (BMI) and 0.949 (waist-to-height ratio), and the coefficients of variation were 1% (BMI) and 3% (waist-to-height ratio).

The abilities of BMI-for-age and waist-to-height ratio to correctly identify children with adverse risk factors are compared in Table 2. The AUCs, positive predictive values, and sensitivities varied substantially across risk factors, with the most accurate classification seen for fasting insulin concentrations. For each
The numbers of children with missing data for the individual risk factors ranged from 0 (SBP and DBP) to 572 (fasting insulin). There were 573 children with missing information on the risk factor sum. SBP, systolic blood pressure; DBP, diastolic blood pressure.

$^{2}$All such values.

$^{3}$Significant difference for sex, $P < 0.001$ (t test).

$^{4}$Significant difference for sex, $P < 0.001$ (t test or Wilcoxon test for triacylglycerols or fasting insulin).

TABLE 1
Characteristics of the subjects

<table>
<thead>
<tr>
<th></th>
<th>Boys ($n = 1501$)</th>
<th>Girls ($n = 1565$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>11 ± 3$^{a}$</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Black (%)</td>
<td>43</td>
<td>44</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.0 ± 4.8</td>
<td>20.2 ± 5.2</td>
</tr>
<tr>
<td>BMI-for-age  z score</td>
<td>0.5 ± 1.1</td>
<td>0.4 ± 1.1</td>
</tr>
<tr>
<td>Overweight (%)</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>68 ± 14$^{a}$</td>
<td>66 ± 12$^{a}$</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>148 ± 20$^{a}$</td>
<td>145 ± 17$^{a}$</td>
</tr>
<tr>
<td>Waist-to-height ratio</td>
<td>0.46 ± 0.06$^{a}$</td>
<td>0.45 ± 0.06$^{a}$</td>
</tr>
<tr>
<td>Total-to-HDL cholesterol ratio</td>
<td>3.2 ± 0.8$^{a}$</td>
<td>3.4 ± 0.9$^{a}$</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)$^{d}$</td>
<td>67 (52, 92)$^{a}$</td>
<td>73 (57, 98)$^{a}$</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>101 ± 25$^{a}$</td>
<td>104 ± 26$^{a}$</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>54 ± 12</td>
<td>53 ± 12</td>
</tr>
<tr>
<td>Fasting insulin (mU/L)$^{d}$</td>
<td>8.6 (6.4, 12.0)$^{a}$</td>
<td>10.0 (7.7, 14.0)$^{a}$</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>103 ± 10$^{a}$</td>
<td>102 ± 10$^{a}$</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>62 ± 9</td>
<td>62 ± 9</td>
</tr>
<tr>
<td>Risk factor sum</td>
<td>0 ± 3</td>
<td>0 ± 3</td>
</tr>
</tbody>
</table>

$^{1}$The numbers of children with missing data for the individual risk factors ranged from 0 (SBP and DBP) to 572 (fasting insulin). There were 573 children with missing information on the risk factor sum. SBP, systolic blood pressure; DBP, diastolic blood pressure.

$^{2}$All such values.

$^{3}$Significant difference for sex, $P < 0.001$ (t test).

$^{4}$Significant difference for sex, $P < 0.001$ (t test or Wilcoxon test for triacylglycerols or fasting insulin).

TABLE 2
Classification of adverse risk factor by BMI-for-age  z score and waist-to-height ratio

<table>
<thead>
<tr>
<th>Risk factor$^{2}$</th>
<th>Area under the curve</th>
<th>Positive predictive value</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMI-for-age  z score</td>
<td>Waist-to-height ratio</td>
<td>BMI-for-age  z score</td>
</tr>
<tr>
<td>Subjects</td>
<td>n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total-to-HDL cholesterol ratio</td>
<td>2961</td>
<td>0.73$^{a}$</td>
<td>0.76$^{a}$</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>2624</td>
<td>0.74</td>
<td>0.75</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>2961</td>
<td>0.61</td>
<td>0.62</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>2961</td>
<td>0.67</td>
<td>0.68</td>
</tr>
<tr>
<td>Fasting insulin</td>
<td>2494</td>
<td>0.81</td>
<td>0.79</td>
</tr>
<tr>
<td>SBP</td>
<td>3066</td>
<td>0.68$^{a}$</td>
<td>0.65$^{a}$</td>
</tr>
<tr>
<td>DBP</td>
<td>3066</td>
<td>0.59</td>
<td>0.60</td>
</tr>
<tr>
<td>Risk factor sum</td>
<td>2493</td>
<td>0.85</td>
<td>0.86</td>
</tr>
</tbody>
</table>

$^{1}$The numbers of children with missing data for the individual risk factors ranged from 0 (SBP and DBP) to 572 (fasting insulin). There were 573 children with missing information on the risk factor sum. SBP, systolic blood pressure; DBP, diastolic blood pressure.

$^{2}$The race-, sex-, and age-adjusted risk factors were used in the analyses.

$^{3}$The cutoff (0.512) for waist-to-height ratio was selected so that equal (17%) proportions of the children would be overweight and would have a high waist-to-height ratio.

$^{4}$Significant difference in area under the curve, $P < 0.05$ as assessed by formulas in Hanely et al (35). The SE of each area under the curve difference was 0.01.
yielded multiple $R^2$ values that were only slightly higher than those obtained with the use of either index. For example, the multiple $R^2$ for the risk factor sum based on both BMI-for-age and waist-to-height ratio was 0.34, whereas the $R^2$ for each index alone was 0.32.

Despite the similarity of the multiple $R^2$ values for the 2 indexes, additional analyses indicated that associations with BMI-for-age were more curvilinear than those with waist-to-height ratio. Predicted measures of several risk factors based on regression models containing either BMI-for-age (left panels) or waist-to-height ratio (right panels) for an 11-y-old white girl are shown in Figure 1. (Predicted measures for boys and black children would be shifted vertically, but they would parallel the curves in Figure 1.) Nonlinearity was most evident in the relation of BMI-for-age to concentrations of triacylglycerol (upper left panel), fasting insulin, and the risk factor sum (bottom left panel) but was also observed for SBP and HDL cholesterol. Furthermore, for each risk factor, the strength of the association increased (steeper slope) at higher measures of BMI-for-age. Associations with waist-to-height ratio (right panels), in contrast, were more linear, and the difference between the 2 indexes was particularly evident for triacylglycerol concentrations. Although waist-to-height ratio showed a nonlinear association ($P < 0.001$) with fasting insulin concentrations, the change in slope was less marked than with BMI-for-age.

We then examined measures of the risk factor sum among children after a cross-classification of categories of BMI-for-age and waist-to-height ratio (Table 4). Waist-to-height ratio measures were categorized so that equal numbers of children would be in each waist-to-height ratio and BMI group. Despite the small number of children in some of the discordant categories (cells above and below the shaded diagonal), the mean risk factor sum tended to increase with measures of both BMI-for-age and waist-to-height ratio. Because of residual confounding, however, these apparent “independent effects” should be interpreted cautiously. A comparison of measures of the risk factor sum in the 2 discordant groups indicated that the mean measure among those who had a high BMI-for-age relative to waist-to-height ratio (6 upper right cells; $\bar{x} = -0.21$) was almost identical to the mean measure among children who had a high waist-to-height ratio relative to BMI-for-age (6 lower left cells; $\bar{x} = -0.19$; $P = 0.88$). Comparable analyses for the individual risk factors indicated that children with a relatively high waist-to-height ratio had slightly

![Figure 1](image.png)

**Figure 1.** The relation of BMI-for-age $z$ score (left) and waist-to-height ratio (right) to risk factor (RF) measures. Predicted measures (for an 11-y-old white girl) were calculated from regression models that included BMI-for-age (or waist-to-height ratio) with age. Continuous variables were modeled by using restricted cubic splines with 5 knots, and the nonlinear effect of BMI-for-age was statistically significant ($P < 0.001$) for each of the 5 RFs. So that the RF sum and fasting insulin could be plotted in the same figure, 9.0 was added to the former. The units for each RF are shown in parentheses. SBP, systolic blood pressure; TG, triacylglycerols.
higher concentrations of total cholesterol: HDL cholesterol and LDL cholesterol, whereas those with a high BMI-for-age had slightly higher concentrations of fasting insulin \((P < 0.05\) for each difference).

Measures of BMI-for-age and waist-to-height ratio for each child, with the triangles representing children who had a high risk factor sum, are shown in Figure 2. The strong association between the 2 indexes is evident, and regression models indicated that measures of BMI-for-age could account for 78% of the variability in measures of waist-to-height ratio. Furthermore, the identification of high measures of the risk factor sum by waist-to-height ratio (horizontal line) and BMI-for-age (vertical line) did not differ. Of the 250 children with a high risk factor sum, 145 (58%) had high measures of both indexes (upper right), 14 (6%) had high measures of BMI-for-age only (lower right), 15 (6%) had high measures of waist-to-height ratio only (upper left), and 76 (30%) did not have high measures of either (lower left).

**DISCUSSION**

Our results show that there is little difference in the abilities of BMI-for-age and waist-to-height ratio to identify children with adverse CVD risk factors. In general, waist-to-height ratio showed slightly stronger associations with lipid and lipoprotein concentrations, whereas BMI-for-age showed slightly stronger associations with measures of fasting insulin and blood pressures. Although some of the differences between the 2 indexes were statistically significant, the AUCs, positive predictive values, sensitivities, and multiple \(R^2\) values were similar for each risk factor. Furthermore, the use of both BMI-for-age and waist-to-height ratio resulted in only slightly better prediction of risk factors than that achieved with only one index. The strong association between BMI-for-age and waist-to-height ratio \((R^2 = 0.78)\) probably accounts for their similar predictive abilities, as well as for the small amount of additional information obtained by using the 2 indexes together.

Various indexes of abdominal obesity (such as waist circumference, waist-to-hip ratio, and waist-to-height ratio) are associated with adverse risk factors among children (13–15) and are predictive of type 2 diabetes and CVD in adulthood (2–8). The limitations of these indexes, however, should be considered. For example, although waist circumference is correlated with the amount of intraabdominal visceral fat, which may be the most detrimental fat depot (9), it is also associated with subcutaneous abdominal fat and with total body fat (38, 39). In addition, a recent study of adults found that waist-to-height ratio and BMI were more strongly associated with each other \((r = 0.85–0.91)\) than with percentage of body fat \((r = 0.69–0.76)\), as determined by air-displacement plethysmography (19). These associations emphasize the potential problems in using waist-to-height ratio and BMI as indexes of abdominal and generalized adiposity, respectively. The interpretation of associations with BMI and waist-to-height ratio is further complicated by the possible relation of disease risk to height (40), which is in the denominator of both indexes.

Some investigators have concluded that, compared with BMI, waist-to-height ratio is more strongly associated with CVD risk factors among children (13–15) and adults (18, 19). It has been emphasized, however, that many of the differences between waist-to-height ratio and BMI are relatively small (19). For example, Hara et al (14) reported that the logarithm of a risk factor score showed correlations of \(r = 0.50\) (waist-to-height ratio) and \(r = 0.45\) (BMI), and Hsieh et al (33) reported correlations of \(r = \ldots\)
0.37 (waist-to-height ratio) and \( r = 0.33 \) (BMI) with a “morbidity index” among men. The slightly stronger relation of BMI-for-age (compared with waist-to-height ratio) to measures of SBP and DBP that we observed was also noted by others (15, 19).

Several explanations are possible for the contrasting findings about the relative importance of BMI-for-age and waist-to-height ratio. Various subsets of risk factors have been included in each study, and only one previous study included an index of insulin resistance (19). (Of the risk factors we examined, fasting insulin concentrations showed the strongest association with BMI.) Furthermore, a study of 36 obese children found that insulin resistance was more strongly associated with total fat mass than with visceral abdominal fat (41). The weaker associations with BMI that were found in previous studies of children may be due to the investigators’ use of BMI rather than BMI-for-age (13, 14) or due to the fact that associations with BMI-for-age were constrained to be linear (14–16, 19). We found that forcing the association with concentrations of fasting insulin to be linear reduced the \( R^2 \) for BMI-for-age from 0.48 (nonlinear) to 0.43 (linear), whereas the \( R^2 \) for waist-to-height ratio decreased from 0.45 only to 0.44. These nonlinear associations may arise because BMI-for-age is a good indicator of adiposity among relatively fat children, but it is an index of both fat and fat-free mass among thinner children (42). If BMI-for-age differences among some relatively thin (eg, BMI-for-age \( z \) score < 1.0) children largely reflect differences in fat-free mass, it would be expected that the relation of BMI-for-age to risk factor measures would be “flatter” (Figure 1) among these children.

The current study has several potential limitations that should be considered. Although the sample was not randomly selected, measures of BMI, lipids, and lipoproteins were fairly comparable to those reported in national studies (29). However, because of differences in methods of measuring blood pressure (28), few children in the Bogalusa Study had an SBP or a DBP > 90th percentile of the National High Blood Pressure Education Program (31). Furthermore, although it can be difficult to compare the magnitudes of the observed associations across studies because of differences in statistical modeling techniques, age ranges, and the specific anthropometric index examined, the magnitudes of the associations that we observed between BMI-for-age and the examined risk factors agree well with those of other studies (43).

Although several prospective studies found the indexes of abdominal obesity to be stronger predictors of CVD and type 2 diabetes than is BMI (4–7), there are conflicting findings. For example, the predictive abilities of BMI and waist-to-height ratio for type 2 diabetes among Pima Indians were almost identical (3), and several studies found that various indexes of abdominal obesity predict disease no better than does BMI (2, 8, 17). For example, the relative risk for coronary heart disease among men in the upper quintile of waist circumference in the Physicians’ Health Study was 1.60, whereas the examined risk factors agree well with those of other studies (43).

In summary, we found that waist-to-height ratio and BMI-for-age showed similar associations with CVD risk factors. Although the use of waist-to-height ratio among children has the potential to simplify the assessment of obesity-related risk, additional information is needed on the tracking of waist-to-height ratios. Furthermore, waist circumference has been measured at numerous sites between the lowest rib and iliac crest, and there are differences between the recommendations of the Anthropometric Standardization Reference Manual (46), the World Health Organization, and the National Institutes of Health (reviewed in reference 45). Small changes in the location of the waist measurement can alter associations with risk factor measures (47–49) and possibly with disease risk.

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Daily food intake in relation to dietary energy density in the free-living environment: a prospective analysis of children born at different risk of obesity$^{1-3}$

Tanja VE Kral, Albert J Stunkard, Robert I Berkowitz, Virginia A Stallings, Danielle D Brown, and Myles S Faith

ABSTRACT

Background: Young children adjust their short-term intake in response to variations in energy density (ED; kcal/g) from preloads in laboratory studies. It remains unknown whether this compensation also occurs under free-living conditions.

Objective: The aims of the study were to test whether children aged 3–6 y regulate their habitual daily food (g) and energy (kcal) intakes in relation to ED and whether compensation differs for children born at different risk of obesity.

Design: Participants were children born at high risk ($n = 22$) or low risk ($n = 27$) of obesity on the basis of maternal prepregnancy body mass index (BMI; in kg/m$^2$). Daily ED, food intake, and energy intake were assessed from 3-d food records that either included or excluded beverages. Intake regulation was explored by relating children’s daily food and energy intakes to ED and, more importantly, by examining residual scores derived by regressing daily food intake on ED.

Results: For both risk groups, daily food intake was inversely correlated with ED ($P < 0.05$), whereas daily energy intake was not significantly correlated with ED at most ages ($P > 0.05$). In analyses that excluded beverages, mean residual scores significantly increased from 3 to 6 y of age in high-risk children, which indicates relative overconsumption, but decreased in low-risk children, which indicates relative underconsumption (risk group × time interaction, $P = 0.005$).


KEY WORDS Energy density, daily energy intake, daily food intake, obesity risk status, children, caloric compensation

INTRODUCTION

The prevalence of childhood obesity is on the rise worldwide (1–10), and the dietary patterns contributing to this increase are poorly understood. One dietary factor that has received less attention in the study of pediatric obesity is dietary energy density (ED), which is defined as calories per weight of food (kcal/g). Controlled laboratory-based feeding studies found that experimentally increasing the ED of foods significantly increases short-term energy intake in adults (11–14). Specifically, across conditions of ED, subjects ate a similar weight of food, thereby increasing their energy intake during a single meal. This suggests that under laboratory conditions, as well as in the free-living environment (15), adults regulate the amount of food they consume (by weight or volume) to a greater extent than the calories they consume.

Studies of children that experimentally altered the ED of foods tested children’s ability to compensate for energy (16–23). Energy compensation refers to the adjustment of food intake during an ad libitum meal in response to variations in the ED from a preload (ie, a fixed amount of food or liquid consumed before the meal). As reviewed by Birch and Fisher (24), most laboratory studies have shown that young children compensate for energy from a preload, although compensation is usually partial and shows considerable interindividual variability. In addition, the effect of compensation ability on longer-term intake regulation (ie, beyond a single laboratory meal) remains poorly understood.

Laboratory protocols have the advantage of assessing children’s food intake objectively. However, they generally limit the food choices, which, in turn, may create conditions that alter the subjects’ habitual eating behavior. Children may compensate reasonably well for energy during a single meal under controlled conditions but not necessarily outside of the laboratory. It is important to assess children’s eating behavior in relation to dietary ED under free-living conditions when they have access to an ample array of foods that vary in ED. It is of particular interest to further elucidate the nature of children’s intake regulation. Specifically, do children regulate the number of calories they consume on a daily basis (25), or do they regulate the amount or the volume of food they ingest, as the data in adults suggest (26–28)? Also, are there differences in intake regulation between children who are born with a different predisposition to obesity and, if so, how does this affect their ability to compensate? It is possible that obesity-promoting genes or family environments influence compensation ability in children. It has also been
suggested that calories consumed from beverages are less well regulated than are those from solid foods (29, 30). Thus, it is possible that the results for children’s intake regulation may differ when liquids (ie, beverages) are included or excluded from the analyses.

A first aim of this study was to examine the interrelation between daily ED (kcal/g), daily food intake (g), and daily energy intake (kcal) among children born at high or low risk of obesity. We predicted that children would reduce their daily food intake as a function of the ED of their diet, thereby regulating daily energy intake. A second aim was to examine children’s daily food intake in relation to their predicted daily food intake for a given level of daily energy density and to assess potential changes in children’s compensation ability over time (from ages 3 to 6 y). We predicted that children’s daily food intakes, relative to their predicted ones, would differ as a function of their obesity risk status. We also predicted that compensation ability would deteriorate over time in both risk groups. All analyses were completed including or excluding all beverages.

SUBJECTS AND METHODS

Subjects

The subjects in this report were part of an ongoing longitudinal study of growth and development in early life that has been conducted at the University of Pennsylvania and the Children’s Hospital of Philadelphia. The children in this study were born at either low or high risk of obesity (31). The children’s obesity risk status was based on maternal prepregnancy body mass index (BMI; in kg/m²). Infants born to mothers with a prepregnancy BMI less than the 33rd percentile (mean BMI of 19.5 ± 1.1) were classified as being at low risk of obesity (n = 37). Children born to mothers with a prepregnancy BMI greater than the 66th percentile (mean BMI of 30.3 ± 4.2) were classified as being at high risk of obesity (n = 35). Children were enrolled in the study at the age of 3 mo, and their growth and development were followed up to year 12. All children in the study were white. Further details of parental and subject characteristics and the study design were reported previously (31–35). The present report is based on a subsample of this cohort (low-risk: n = 27; high-risk: n = 22) for whom 3-d weighed-food records were available at 3, 4, 5, and 6 y of age. Written informed consent was obtained from the parents. The protocol was approved by the institutional review boards of the University of Pennsylvania and the Children’s Hospital of Philadelphia.

Dietary assessment

Each year, within 2 wk of each child’s birthday, the primary caretakers of children in both risk groups were asked to complete 3-d weighed-food records (2 weekdays, 1 weekend day). Caretakers were provided electronic food scales to preweigh all foods and beverages (except water) consumed by the child and to weigh all leftovers. Food records were analyzed by research nutritionists at the General Clinical Research Center at the Children’s Hospital of Philadelphia by using the Food Processor Nutrition Analysis software (ESHA Research, Salem, OR). Only those food records that were completed for ≥2 d were included in the present analyses. Two- and three-day food records were available for the following numbers of children at ages 3, 4, 5, and 6 y: 45 (40 three-day records and 5 two-day records); 48 (45 three-day records and 3 two-day records); 42 (38 three-day records and 4 two-day records); and 42 (39 three-day records and 3 two-day records), respectively. One three-day food record was not included in the analyses because the child was fed infant formula almost exclusively. Likewise, 2 one-day records were not included because the children were either sick or fell asleep before dinner.

Dietary outcome measures

The following 3 dietary outcome measures were computed from the 3-d weighed-food records: daily ED (kcal/g), daily food intake (g), and daily energy intake (kcal). All 3 outcome variables were computed either to include or exclude all beverages. The daily dietary ED was computed by dividing the total daily calories by the total amount of food and beverages (ie, weight in grams) consumed (36). Daily food and energy intakes were computed as the sums of the individual weights and calories consumed from foods and beverages on a given day. All dietary outcome measures were averaged across the total number of days of completed records included in the analyses. A detailed description of the children’s daily energy density and daily energy intake is provided elsewhere (37).

Statistical analysis

Descriptive statistics are presented as means ± SEMs. To test aim one, a Pearson correlation analysis examined relations between daily ED and daily energy and food intakes. Dietary ED was correlated first with daily food intake and second with daily energy intake within each risk group for each year. These correlation analyses were completed by using intakes that included or excluded all beverages.

Because the correlations tested in aim one could have been due in part to the functional relation between these variables (38), we proceeded by using an analysis of residuals to further examine children’s intake regulation. Thus, to test the second aim of the study, an analysis of residuals was conducted in which, for each age, daily food intake was regressed onto daily ED and the residual scores were saved. The residual is that part of daily food intake that is uncorrelated with daily ED. Residuals are the differences between an observed value of the response variable and the value predicted by the model. In our model, the residuals represent the vertical difference between the regression line (or the predicted value) and the actual (or observed) data. The residual represents the difference between an individual’s actual food intake and what their food intake would be expected to be for a given level of ED based on this sample. Thus, the residuals allow us to break down the sample into children who eat more or less than would be expected. Positive residuals represent greater food consumption than would be expected (ie, overconsumption), whereas negative residuals represent less food consumption than expected (ie, underconsumption). A residual of zero represents what the model would predict food intake to be for a given ED value on the basis of this sample; thus, the deviation from the perfect prediction is zero.

A 2 (risk group) × 4 (age) mixed linear model analysis of variance (ANOVA) tested for (linear) differences in the mean residual food scores as a function of risk group and child age.
TABLE 1
Daily energy intake, food intake, and energy density stratified by age and risk group

<table>
<thead>
<tr>
<th>Energy intake, food only (kcal)²</th>
<th>Age 3 y</th>
<th>Age 4 y</th>
<th>Age 5 y</th>
<th>Age 6 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low risk</td>
<td>898 ± 47</td>
<td>939 ± 55</td>
<td>1064 ± 52</td>
<td>1159 ± 45</td>
</tr>
<tr>
<td>High risk</td>
<td>847 ± 51</td>
<td>965 ± 57</td>
<td>1177 ± 70</td>
<td>1393 ± 67³</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Energy intake, food and beverages (kcal)⁴</th>
<th>Age 3 y</th>
<th>Age 4 y</th>
<th>Age 5 y</th>
<th>Age 6 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low risk</td>
<td>1145 ± 49</td>
<td>1219 ± 62</td>
<td>1311 ± 58</td>
<td>1435 ± 43</td>
</tr>
<tr>
<td>High risk</td>
<td>1189 ± 59</td>
<td>1253 ± 61</td>
<td>1493 ± 84</td>
<td>1687 ± 69</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Food intake, food only (g)⁵</th>
<th>Age 3 y</th>
<th>Age 4 y</th>
<th>Age 5 y</th>
<th>Age 6 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low risk</td>
<td>443 ± 25</td>
<td>451 ± 30</td>
<td>466 ± 25</td>
<td>525 ± 31</td>
</tr>
<tr>
<td>High risk</td>
<td>395 ± 24</td>
<td>447 ± 24</td>
<td>526 ± 34</td>
<td>620 ± 36</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Food intake, food and beverages (g)⁶</th>
<th>Age 3 y</th>
<th>Age 4 y</th>
<th>Age 5 y</th>
<th>Age 6 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low risk</td>
<td>948 ± 45</td>
<td>1018 ± 58</td>
<td>980 ± 54</td>
<td>1104 ± 42</td>
</tr>
<tr>
<td>High risk</td>
<td>1062 ± 74</td>
<td>1097 ± 58</td>
<td>1210 ± 90</td>
<td>1262 ± 51</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Energy density, food only (kcal/g)⁷</th>
<th>Age 3 y</th>
<th>Age 4 y</th>
<th>Age 5 y</th>
<th>Age 6 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low risk</td>
<td>2.14 ± 0.10</td>
<td>2.23 ± 0.08</td>
<td>2.37 ± 0.09</td>
<td>2.31 ± 0.08</td>
</tr>
<tr>
<td>High risk</td>
<td>2.23 ± 0.09</td>
<td>2.24 ± 0.11</td>
<td>2.36 ± 0.11</td>
<td>2.34 ± 0.10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Energy density, food and beverages (kcal/g)⁸</th>
<th>Age 3 y</th>
<th>Age 4 y</th>
<th>Age 5 y</th>
<th>Age 6 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low risk</td>
<td>1.26 ± 0.06</td>
<td>1.27 ± 0.05</td>
<td>1.39 ± 0.06</td>
<td>1.35 ± 0.06</td>
</tr>
<tr>
<td>High risk</td>
<td>1.21 ± 0.07</td>
<td>1.19 ± 0.05</td>
<td>1.30 ± 0.07</td>
<td>1.39 ± 0.09</td>
</tr>
</tbody>
</table>

¹ All values are ± SEM. These data were part of a previous publication by Kral et al (37).
² 2 x 4 ANOVA indicated a significant (linear) risk group × time interaction (P < 0.02).
³ Significantly different from the low-risk group, P < 0.05.
⁴ 2 x 4 ANOVA indicated a significant main and linear effect of time (P < 0.0001) but no significant risk group × time interaction (P = 0.19).
⁵ 2 x 4 ANOVA indicated a significant risk group × time interaction (P = 0.02).
⁶ 2 x 4 ANOVA indicated a significant linear effect of time (P < 0.0001) but no risk group × time interaction (P = 0.40).
⁷ 2 x 4 ANOVA indicated a significant linear effect of time (P = 0.03) but no risk group × time interaction (P = 0.98).
⁸ 2 x 4 ANOVA indicated a significant linear effect of time (P = 0.002) but no risk group × time interaction (P = 0.58).

Risk group was a between-subjects variable with 2 levels (low-risk or high-risk), and child age was a within-subjects variable with 4 levels (3, 4, 5, or 6 y). A main effect of risk group would imply that the tendency to overconsume food differs for high-risk and low-risk children. Because of the repeated-measures structure of the data, the model used restricted maximum likelihood estimates and a compound symmetry error structure. Significant main effects or interactions were followed up by pair-wise comparisons, as well as contrasts, to test for potential linear effects of time across all 4 ages and linear time by risk group interactions. Again, these analyses were completed by using intakes that included or excluded all beverages.

The ANOVA model to test aim 2 was run with and without child BMI z score as a covariate to ensure that any risk group differences in residual scores were not merely due to differences in children’s weight status. We identified 2 children in the low-risk group who, at age 4 y, qualified as statistical outliers per Tukey’s criteria (39) with regard to BMI z score within their respective risk group. We comment in the Results section that the outcomes did not change when the outliers were removed.

We adjusted the α level by using the Bonferroni correction (ie, α = 0.05/n, where n equals the number of comparisons in a given analysis). We note associations that were significant both at the conventional α = 0.05 level as well as when using the Bonferroni correction because this correction can be overly stringent (40). We used the same approach in previous analyses of this cohort (33).

The data were analyzed by using SPSS software (version 12.0; SPSS Inc, Chicago, IL) and SAS software (version 9.1; SAS Institute, Inc, Cary, NC). For all analyses, P values < 0.05 were considered statistically significant.

RESULTS

Child characteristics

The number of low-risk children included in the present study at ages 3, 4, 5, and 6 y were 23 (13 boys, 10 girls), 27 (14 boys, 13 girls), 23 (11 boys, 12 girls), and 22 (8 boys, 14 girls); the number of high-risk children were 22 (12 boys, 10 girls), 21 (9 boys, 12 girls), 19 (10 boys, 9 girls), and 20 (11 boys, 9 girls), respectively. The mean (±SEM) BMI z scores for high-risk children were −0.4 ± 0.3, 0.4 ± 0.3, 0.5 ± 0.4, and 0.2 ± 0.3, and those for low-risk children were −0.4 ± 0.2, 0.1 ± 0.1, 0.1 ± 0.2, and −0.3 ± 0.2 at ages 3, 4, 5, and 6 y, respectively. Student t tests showed that, at each of the years, none of these BMI z scores were significantly different between risk groups. More detailed analyses of anthropometric measures for this subsample (37) as well as the full cohort (34) are provided elsewhere.

Daily food intake, energy intake, and energy density

The mean daily food intake (g), daily energy intake (kcal), and daily energy density (kcal/g) for children in both risk groups at ages 3, 4, 5, and 6 y are shown in Table 1. These data are displayed for descriptive purposes only. A detailed discussion of these results can be found in a previously published paper by Kral et al (37) that investigated the changes in dietary energy density in children in this cohort over time.
Daily energy intake, however, was not significantly correlated with daily food intake as a function of ED during these years. All beverages included

Daily food intake was significantly inversely correlated with daily ED for the low-risk group at all ages and for the high-risk group at ages 5 and 6 y ($P < 0.05$; Table 2). The inverse relation was borderline significant in the high-risk group at 3 y of age ($P = 0.06$). This indicates that children who consumed a diet that was relatively higher in ED tended to eat less food on a daily basis than did children who consumed a diet that was relatively lower in ED. Two of these 8 associations remained significant, and 2 were borderline significant, when applying a Bonferroni-adjusted alpha ($0.05/8 = 0.006$).

Daily energy intake was not significantly correlated with daily ED for the low-risk group at all ages and for the high-risk group at ages 3, 5, and 6 y (Table 2). This indicates that across a range of dietary ED, daily caloric intake did not vary significantly as a function of ED during these years.

Relation between daily food intake and energy density and between daily energy intake and energy density

All beverages excluded

Daily food intake was significantly inversely correlated with daily ED for the low-risk group at all ages and for the high-risk group at ages 5 and 6 y ($P < 0.05$; Table 2). The inverse relation was borderline significant in the high-risk group at 3 y of age ($P = 0.06$). This indicates that children who consumed a diet that was relatively higher in ED tended to eat less food on a daily basis than did children who consumed a diet that was relatively lower in ED. Two of these 8 associations remained significant, and 2 were borderline significant, when applying a Bonferroni-adjusted alpha ($0.05/8 = 0.006$).

Daily energy intake was not significantly correlated with daily ED for the low-risk group at all ages and for the high-risk group at ages 3, 5, and 6 y (Table 2). This indicates that across a range of dietary ED, daily caloric intake did not vary significantly as a function of ED during these years.

All beverages included

Daily food intake was significantly inversely correlated with daily ED for both the low-risk and the high-risk group at all ages ($P < 0.05$; Table 3). Six of these 8 associations remained significant when applying a Bonferroni-adjusted alpha ($0.05/8 = 0.006$). Daily energy intake, however, was not significantly correlated with daily ED for either group at any age (Table 3).

Residual analysis

Mean residual food intake scores for each risk group by child age are displayed in Figure 1. The 2 (risk group) × 4 (age) ANOVA indicated a significant risk group × age category interaction ($P = 0.005$) and a linear risk group × age (as a continuous variable) interaction ($P = 0.0005$). This interaction was still significant when applying a Bonferroni-adjusted alpha in the respective ANOVA models ($0.05/3 = 0.02$). Specifically, mean residual scores increased between ages 3 and 6 y among high-risk children, reflecting a greater tendency to overconsume food relative to predicted intake, but decreased among low-risk children, reflecting a greater tendency to underconsume food relative to predicted intake. A pairwise follow-up comparison indicated that the mean residual food intake score was significantly greater in the high-risk children than in the low-risk children at age 6 y (residual $= 53.4 \pm 137.0$ versus $-48.5 \pm 105.1; P = 0.01$).

The risk group × age interaction remained significant ($P = 0.02$) when BMI $z$ score was added to the model as a covariate. The main effect of BMI $z$ score was significant in this model ($P = 0.04$), indicating that heavier children tended to overconsume food (relative to their predicted intake) compared with thinner children. After removal of the 2 statistical outliers for BMI $z$ score from the model, the main effect of BMI $z$ score was not significant ($P = 0.06$); however, the risk group × age interaction remained significant ($P = 0.02$). We note that this

### TABLE 2
Pearson correlation coefficients summarizing the relation between dietary energy density and daily food and energy intakes (excluding beverages) by risk group and child age

<table>
<thead>
<tr>
<th>Risk group</th>
<th>Daily intake</th>
<th>Age 3 y</th>
<th>Age 4 y</th>
<th>Age 5 y</th>
<th>Age 6 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low risk</td>
<td>Food intake, food only (g)</td>
<td>$-0.55^2$</td>
<td>$-0.49^2$</td>
<td>$-0.50^3$</td>
<td>$-0.70^2$</td>
</tr>
<tr>
<td>High risk</td>
<td>Food intake, food only (g)</td>
<td>$-0.40^2$</td>
<td>$-0.23$</td>
<td>$-0.65^2,^4$</td>
<td>$-0.51^2$</td>
</tr>
<tr>
<td>Low risk</td>
<td>Energy intake, food only (kcal)</td>
<td>$0.31$</td>
<td>$0.10$</td>
<td>$0.30$</td>
<td>$-0.01$</td>
</tr>
<tr>
<td>High risk</td>
<td>Energy intake, food only (kcal)</td>
<td>$0.21$</td>
<td>$0.50^4$</td>
<td>$0.13$</td>
<td>$0.29$</td>
</tr>
</tbody>
</table>

$^1$ With the use of Fisher $r$-to-$z$ transformations and a 2-tailed significance test, none of the correlation coefficients was significantly different within each age group or across age groups.

$^2$ Correlation significant at 0.01 level.

$^3$ Correlation significant at 0.05 level.

$^4$ $P < 0.006$ (Bonferroni correction, $\alpha = 0.05/8$).

$^5$ $P = 0.06$.

### TABLE 3
Pearson correlation coefficients summarizing the relations between dietary energy density and daily food and energy intakes (including beverages) by risk group and child age

<table>
<thead>
<tr>
<th>Risk group</th>
<th>Daily intake</th>
<th>Age 3 y</th>
<th>Age 4 y</th>
<th>Age 5 y</th>
<th>Age 6 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low risk</td>
<td>Food, food, and beverages (g)</td>
<td>$-0.58^2,^3$</td>
<td>$-0.45^4$</td>
<td>$-0.64^2,^3$</td>
<td>$-0.78^2,^3$</td>
</tr>
<tr>
<td>High risk</td>
<td>Food, food, and beverages (g)</td>
<td>$-0.69^2,^4$</td>
<td>$-0.49^4$</td>
<td>$-0.62^2,^3$</td>
<td>$-0.61^2,^3$</td>
</tr>
<tr>
<td>Low risk</td>
<td>Energy intake, food, and beverages (kcal)</td>
<td>$0.28$</td>
<td>$0.13$</td>
<td>$-0.05$</td>
<td>$0.26$</td>
</tr>
<tr>
<td>High risk</td>
<td>Energy intake, food, and beverages (kcal)</td>
<td>$-0.002$</td>
<td>$0.40$</td>
<td>$0.13$</td>
<td>$0.42$</td>
</tr>
</tbody>
</table>

$^1$ With the use of Fisher $r$-to-$z$ transformations and a 2-tailed significance test, none of the correlation coefficients was significantly different within each age group or across age groups.

$^2$ Correlation significant at 0.01 level.

$^3$ $P < 0.006$ (Bonferroni correction, $\alpha = 0.05/8$).

$^4$ Correlation significant at 0.05 level.
interaction effect approached but did not reach significance when using the Bonferroni-adjusted $\alpha (0.05/4 = 0.01)$.

**All beverages included**

Mean residual food intake scores for each risk group by child age are displayed in **Figure 2**. The 2 (risk group) × 4 (age) ANOVA indicated a trend for a main effect of risk group ($P = 0.07$), which suggests a tendency for high-risk children to overconsume, relative to their predicted intake, compared with low-risk children across all 4 y. The risk group × age interaction was not significant ($P = 0.55$), nor was the linear risk group × age interaction ($P = 0.38$). None of the main effects or the interaction was significant when using a Bonferroni-adjusted $\alpha (0.05/3 = 0.02)$.

**DISCUSSION**

The main finding of the present study was that across a range of ED, children born at high or low risk of obesity adjusted the daily amount of food they consumed on the basis of the ED of their diet. Children who consumed a diet that was higher in ED consumed less food daily than did children whose diet was lower in ED. These findings held true regardless of whether beverages were included or excluded. This is the first longitudinal study to show that children aged 3–6 y also adjust their daily intake under free-living conditions. These findings thus confirm and extend previous research conducted under laboratory conditions.

The prospective nature of this design also allowed us to test for changes in energy regulation ability over time. It has been suggested that developmental differences in energy compensation ability may exist in children (24). Preschool children, on average, show compensation (24); however, Anderson et al (41) found no effect on food intake when varying the ED of preloads in 9–10-y-old children. These findings indirectly suggest developmental changes in compensation ability; however, this phenomenon has never been documented in long-term prospective studies that track children during growth.

With the use of a novel approach of residual score analysis, this present study assessed changes in compensation ability over the course of 4 y. The results indicated different patterns of residuals between high- and low-risk children, which suggests that familial predisposition to obesity may partially operate through errors in compensation. This supports theories and experimental studies regarding energy compensation ability and the development of pediatric obesity (22–24, 42). However, it should be pointed out that the results from the residual analysis are sample-dependent and therefore may not apply to other cohorts of children. The main finding of the residual analysis was that high-risk children tended to overconsume, whereas low-risk children tended to underconsume, relative to their predicted intake, although the results differed depending on whether beverages were included in the analyses. For analyses that excluded beverages, the risk group differences gradually emerged over time, with high-risk children not showing a tendency to overconsume until after 4 y of age. This is consistent with the notion that compensation ability gradually deteriorates during early childhood, possibly starting during the years corresponding to adiposity rebound (43), rather than being compromised consistently and as early as 3 y of age. This gradual decline in compensation ability was not seen when beverages were included in the analyses (see below). Another key finding was that the risk group differences remained significant even when the analyses were controlled for BMI $z$ score, which suggests that the errors in compensation cannot be fully explained by differences in weight status. Again, this was not the case when beverages were included in the analyses (see below).

When beverages were included in the residual score analyses, the results indicated a trend ($P = 0.07$) for high-risk children to...
overconsume and low-risk children to underconsume across all 4 y. That is, errors in compensation did not appear to gradually emerge over time but may have been present as early as 3 y of age. This interpretation, if true, would suggest that errors in compensation ability for (food and) beverages are established by 3 y of age and, therefore, that any deterioration in compensation ability may have occurred before the age of 3 y. This finding raises the possibility that infancy and the first 2 y of life may be critical periods for the development of compensation ability for food and beverages (20, 21, 44, 45) and therefore may be important periods for early-life interventions. To more convincingly identify critical periods of time when children’s ability to compensate may start to deteriorate, however, studies are needed that examine (or track) the stability of compensation indexes. Findings from the current study are also consistent with studies reporting that excess beverage consumption (especially from fruit juice) is associated with excess weight gain in preschool-age children (46, 47). Hunger and thirst mechanisms are believed to be distinct entities, because the consumption of beverages primarily acts on thirst and not hunger mechanisms (48). It is possible that liquids, which are thought of as having a generally lower satiating efficiency (49), may thus further weaken children’s compensation ability.

Another unique finding of the residual score analyses that included beverages was that the risk group differences no longer approached statistical significance when BMI z score was added as a covariate. BMI z score was consistently associated with greater residual scores across analyses, which suggests that errors in compensation ability for beverages (and food) may be affected by child weight status and the determinants thereof. Heavier children may be more likely to exceed their energy needs through consumption of beverages (46). Despite these findings, it should be noted that beverages, because of their low energy density, add weight but fewer calories to the daily total, and, thus, by computation, disproportionately influence dietary ED. Hence, residual analyses that include beverages may need to be interpreted with more caution.

The result of this investigation agrees with findings from 2 recent experimental studies (22, 23) that showed that energy compensation in children J) is often incomplete and shows great interindividual variability and 2) tends to decline with age. It is possible that children, especially those who are genetically predisposed to obesity, may become more responsive to environmental influences (such as the portion size of foods) as they become older. A study conducted by Rolls et al (50) showed that when presented with larger portions of food, 5-y-old children, but not 3-y-old children, significantly increased their intakes relative to when they were presented with smaller portions of food. The authors suggested that as children age they become increasingly responsive to environmental, social, and cultural factors that all are likely to affect their intake. To what extent these factors increase children’s vulnerability to overeat may depend on parental obesity, a line of research that has yet to be further explored.

The strengths of the present study include 1) the unique sample of children born at different risk of obesity and who were prospectively studied and 2) the use of extensive training and electronic food scales to measure the weights of the foods that the children consumed. The study might well be extended in several ways. First, it would be desirable to examine ability to adjust food intake in relation to dietary ED in a larger cohort of children who are ethnically diverse and who show greater variability in their weight status and age than did the children in the current cohort. Thus, the results from this study are limited to the relatively narrow study sample, namely, healthy, full-term, white children, and may not be generalizable to children as a whole. Second, it would be desirable to obtain intake data over a longer period of time at each age to assess children’s habitual diet. Third, the validity of the data may be increased by complementing self-reported intakes, which may be subject to measurement or reporting errors by mothers, with measured intake data in the feeding laboratory. Fourth, future research should also collect data of energy expenditure to be able to relate children’s intake data to their energy needs. Last, the present design, unlike a classic twin design or other more genetically sensitive designs, could not formally test the respective influence of genetic and environmental influences on compensation ability.

In conclusion, the results of the present study suggest that children between the ages of 3 and 6 y have the ability to adjust their daily food intake on the basis of the ED of their diet to maintain a certain daily energy intake in the free-living environment. Energy compensation ability, however, may differentiate children who were born at high-risk or at low-risk of obesity. Future research is needed that separates the effects of food and beverages on children’s compensation ability.

We thank the General Clinical Research Center of the Children’s Hospital of Philadelphia and the staff of the Infant Growth Study for their support. We also thank Michael J Rovine for statistical consulting on the study.

The contributions of the authors were as follows—TVEK: contributed to the study design, data acquisition and analysis, and writing of the manuscript; AJS, RJB, VAS, and MSF: were responsible for the study concept, acquisition of the data, writing of the manuscript, and obtaining funding; DDB: contributed to the data analysis. None of the authors had any conflicts of interest.

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Abdominal obesity and coronary artery calcification in young adults: the Coronary Artery Risk Development in Young Adults (CARDIA) Study

Chong-Do Lee, David R Jacobs Jr, Pamela J Schreiner, Carlos Iribarren, and Arlene Hankinson

ABSTRACT
Background: Whether abdominal obesity is related to coronary artery calcification (CAC) is not known.
Objective: We investigated the relations of waist girth and waist-hip ratio (WHR) to CAC in 2951 African American and white young adults from the Coronary Artery Risk Development in Young Adults Study.
Design: The present study was a cross-sectional and observational cohort study. Using standardized protocols, we measured CAC in 2001–2002 by using computed tomography and measured waist and hip girths in 1985–1986 (baseline), 1995–1996 (year 10), and 2001–2002 (year 15, waist girth only). CAC was classified as present or absent, whereas waist girth and WHR were placed in sex-specific tertiles.
Results: After adjustment for age, sex, race, clinical center, physical activity, cigarette smoking, education, and alcohol intake, baseline waist girth and WHR were directly associated with a higher prevalence of CAC 15 y later (P for trend < 0.001 for both). The odds ratios (ORs) for CAC in the highest versus lowest tertiles of waist girth and WHR were 1.9 (95% CI: 1.36, 2.65) and 1.7 (1.23, 2.41), respectively. Waist girth and WHR at year 10 and waist girth at year 15 similarly predicted CAC. These associations persisted after additional adjustment for systolic blood pressure, fasting insulin concentrations, diabetes, and antihypertensive medication use but became nonsignificant after additional adjustment for blood lipids.
Conclusions: Abdominal obesity measured by waist girth or WHR is associated with early atherosclerosis as measured by the presence of CAC in African American and white young adults. This is consistent with an involvement of visceral fat in the occurrence of coronary artery calcium in young adults. Am J Clin Nutr 2007; 86:48–54.

KEY WORDS Coronary artery calcification, abdominal obesity, waist girth, waist-hip ratio

INTRODUCTION
Atherosclerosis is a major cause of coronary heart disease (CHD) and ischemic stroke. The progression of plaque formation and calcium deposition is associated with the accumulation of macrophages, smooth muscle cells, fibrosis, necrosis, and lipids in the arteries (1, 2). Coronary artery calcification (CAC) is a risk marker for atherosclerosis and is positively associated with CHD and cardiovascular disease (CVD) events (3–6). In general, the established risk factors for coronary calcification are the same as those for clinical CVD: male sex, age, body mass index, elevated blood pressure, diabetes mellitus, cigarette smoking, and LDL and HDL cholesterol (3, 7–9).

Abdominal obesity is also a significant risk factor for athero-sclerosis. Excessive accumulation of visceral fat is associated with insulin resistance and compensatory hyperinsulinenia, which contributes to atherosclerotic progression (10). An elevated insulin concentration is a risk marker for dyslipidemia and hypertension, and it promotes smooth muscle cell proliferation and cholesterol ester accumulation in the artery (11). However, there has been little research on the relation between abdominal obesity and subclinical atherosclerotic vascular disease across race, sex, and age groups in population-based studies. We therefore investigated the relation of visceral fat measured by waist girth or waist-hip ratio (WHR) to CAC in young African American and white men and women from the Coronary Artery Risk Development in Young Adults (CARDIA) Study.

SUBJECTS AND METHODS
Study population
The CARDIA study is a population-based cohort study designed to investigate the causes of atherosclerosis in a young biracial population from 4 communities in the United States: Birmingham, AL; Chicago, IL; Minneapolis, MN; and Oakland, CA. The baseline study population comprised 5115 African American and white men and women aged 18–30 y, who were recruited in 1985 and 1986. The participants were selected according to a balanced design by race, sex, educational attainment

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2 The Coronary Artery Risk Development in Young Adults (CARDIA) Study was supported by contracts N01-HC-48047, N01-HC-48048, N01-HC-48049, N01-HC-48050, N01-HC-95095, and N01-HC-45134 from the National Heart, Lung, and Blood Institute.
3 Reprints not available. Address correspondence to C-D Lee, Department of Exercise and Wellness, Arizona State University, 7350 East Unity Avenue, Mesa, AZ 85212. E-mail: chong.lee@asu.edu.
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(less than high school, completion of high school, or more than high school), and age groups (18–24 and 25–30 y old). The complete study design, sampling strategy, and examination techniques were reported previously (12). The participants were re-examined during 1987–1988 (year 2), 1990–1991 (year 5), 1992–1993 (year 7), 1995–1996 (year 10), and 2000–2001 (year 15), when the rate of retention was 90%, 86%, 81%, 79%, and 74%, respectively. The current study is based on those 3043 persons who underwent a coronary artery scan at the year 15 examination to determine the presence or absence of calcium deposits in the arteries.

All subjects gave written informed consent for the clinical examination. The institutional review boards at all sites approved the protocol.

Measurements

All participants were asked to fast for 12 h before the clinical examination. Body height and weight were measured with a calibrated scale and a vertical ruler, respectively, and body mass index (BMI; in kg/m²) was calculated. Waist girth was measured laterally at the point midway between the iliac crest and the lowest lateral portion of the rib cage and anteriorly at the point midway between the xiphoid process of the sternum and the umbilicus. The hip girth was measured anteriorly at the level of the symphysis pubis and posteriorly at the level of the maximal protrusion of the gluteal muscles by using a spring-gauge plastic tape measure. Seated blood pressure was measured after 5 min of rest with the use of a random-zero sphygmomanometer, and the average of the last 2 of 3 consecutive measurements was used for analysis. Serum, plasma, and whole blood samples were drawn from an antecubital vein. Plasma concentrations of total cholesterol, HDL cholesterol, and triacylglycerol were measured with an enzymatic method (13). HDL cholesterol was measured after dextran-magnesium precipitation (14), and LDL cholesterol was calculated by using the Friedewald equation (15). Fasting insulin was measured with the use of a radioimmunoassay (Linco Research, St Charles, MO). Test-retest reliability coefficients for total, HDL, and LDL cholesterol and triacylglycerol were >0.98 (16). Additional details of examination procedures were published previously (16, 17).

CAC was determined at the year 15 examination by using computed tomography (CT). Trained technicians scanned the root of aorta to the apex of the heart and obtained 40 contiguous 2.5–3.0-mm-thick transverse images by using electron beam CT (Chicago and Oakland field centers) and multidetector CT (Birmingham and Minneapolis field centers). All participants were scanned twice over a hydroxyapatite phantom to allow monitoring of image brightness and noise and adjustment for scanner differences. Scans were electronically sent to the CARDIA Reading Center, and a radiologist identified the courses of the coronary arteries by using specially developed image-processing software (developed at the Harbor-UCLA Medical Center CT Reading Center, Los Angeles, CA) programmed to define a calcific focus as 4 adjacent pixels composing an area of ≥1.87 mm². A calcium score was calculated for each calcified lesion by multiplying the area of focus by a coefficient based on the peak CT number in the focus. The coefficient ranged from 1 to 4 [1 = 131–200 Hounsfield units (HU), 2 = 201–300 HU, 3 = 301–400 HU, and 4 = ≥401 HU]. Total calcium scores were obtained by summing all lesions within a given artery and across all arteries (left main, left anterior descending, left circumflex, and right coronary arteries). Each scan set with ≥1 nonzero coronary calcium score and a random sample of those with 0 score were reviewed by an expert investigator who had no knowledge of the scan scores, to verify the presence of CAC. The overall score was calculated as the average of the 2 scans if the investigator adjudicated the scans as being positive and was set to zero for those scans adjudicated as negative. Details of the CAC examination techniques and procedures were published previously (18).

Cigarette smoking, alcohol intake, physical activity, and educational level were assessed by means of standardized questionnaires. Smoking status was classified as never smoker, former smoker, or current smoker, and alcohol intake was classified as a continuous variable (ethanol intake, in mL/d). Self-reported physical activity scores were computed by multiplying the frequency of participation by the intensity of the activity (19). Educational level was classified by the number of years of education: less than high school, completion of high school, or at least some college. The use of antihypertensive or cholesterol-lowering medication was assessed by the self-reported questionnaire. Diabetes mellitus was defined as a fasting glucose concentration ≥126 mg/dL or the use of hypoglycemic agents.

Statistical analysis

After the exclusion of 4 pregnant women at baseline, 2 pregnant women at year 15 examination, and subjects who were missing dependent or independent variables or covariate values, we included 2951 men and women aged 33–45 y who underwent a coronary artery scan at the year 15 examination. Coronary calcium scores were classified as dichotomous variable (0 = absence; 1 = presence if calcium score was >0), and the waist girth and WHR were classified by using sex-specific tertiles. General linear models were used to test mean differences across waist girth categories after adjustment for age, sex, race, and field center. The chi-square test was used to compare frequency differences across waist girth categories. Multivariate logistic regression models were used to investigate the associations of waist girth (baseline, year 10, and year 15) and WHR (baseline and year 10) with the year 15 presence of CAC after adjustment for age, sex, race, and clinical center (model 1); after additional adjustment for physical activity, cigarette smoking, education, and alcohol intake (model 2); after additional adjustment for systolic blood pressure, fasting insulin, diabetes, and antihypertensive medication use from model 2 (model 3); and after additional adjustment for blood lipids (HDL and total cholesterol and triacylglycerol) from model 3 (model 4). The additional adjustment variables in models 3 and 4 could be in the causal pathway between waist girth and CAC; we regarded these models as explanatory, rather than deconfounding. The smallest waist girth or the lowest WHR tertile was the reference category. Trends across exposure categories were tested by treating those categories as a categorical scale with adjustment for covariates. We also examined the race- and sex-adjusted partial Pearson correlations among waist girth, WHR, and BMI across baseline, year 10, and year 15 examinations. All statistical procedures were performed by using SAS software (version 9; SAS Institute, Cary, NC).

RESULTS

As shown in Table 1, the prevalences or means of several baseline variables differed according to baseline waist girth. Prevalences of diabetes and antihypertensive medication use
TABLE 1
Characteristics of the study participants across waist girth categories at baseline and coronary artery calcification at year 15: the CARDIA Study, 1985–2001

<table>
<thead>
<tr>
<th>Sex-specific waist girth tertiles²</th>
<th>1 (n = 968)</th>
<th>2 (n = 994)</th>
<th>3 (n = 989)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>24.3</td>
<td>25.3</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>African American (%)</td>
<td>43.9</td>
<td>41.5</td>
<td>50.1</td>
<td></td>
</tr>
<tr>
<td>Adjusted values¹⁴</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.8</td>
<td>23.4</td>
<td>28.8</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Systolic blood pressure (mg/dL)</td>
<td>108.4</td>
<td>109.9</td>
<td>113.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>172.4</td>
<td>176.7</td>
<td>183.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>56.8</td>
<td>54.4</td>
<td>48.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>103.7</td>
<td>109.1</td>
<td>117.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Triacylglycerol (mg/dL)</td>
<td>60.0</td>
<td>66.3</td>
<td>89.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fasting insulin (µU/mL)</td>
<td>7.6</td>
<td>9.0</td>
<td>14.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Alcohol intake (mL/d)</td>
<td>11.2</td>
<td>11.6</td>
<td>13.8</td>
<td>0.01</td>
</tr>
<tr>
<td>Physical activity score</td>
<td>427.3</td>
<td>446.9</td>
<td>401.2</td>
<td>0.002</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>0.1</td>
<td>0.4</td>
<td>1.1</td>
<td>0.008</td>
</tr>
<tr>
<td>Antihypertensive medication use (%)</td>
<td>0.4</td>
<td>1.7</td>
<td>4.0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Current smoking (%)</td>
<td>27.0</td>
<td>23.5</td>
<td>28.8</td>
<td>0.03</td>
</tr>
<tr>
<td>Educational level (&lt; high school) (%)</td>
<td>8.7</td>
<td>5.1</td>
<td>7.9</td>
<td>0.006</td>
</tr>
<tr>
<td>Year 15 outcome variable</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary artery calcification (%)</td>
<td>6.4</td>
<td>8.0</td>
<td>13.8</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

¹ n = 2951. CARDIA, Coronary Artery Risk Development in Young Adults. All characteristics except coronary artery calcification were measured at CARDIA year 0 when the participants were aged 18–30 y.

² Waist girth cutoffs: <77.5, 77.5–<84.3, and ≥84.3 cm (men) and <68, 68–<75.5, and ≥75.5 cm (women) for tertiles 1, 2, and 3, respectively.

³ Values are X̄.

⁴ Adjusted for age, sex, race, and clinical center.

were progressively higher across rising sex-specific waist girth tertiles. Means of BMI, systolic blood pressure, LDL and total cholesterol, triacylglycerol, fasting insulin, and alcohol intake were also progressively higher, and that of HDL cholesterol was lower across rising waist girth categories. Physical activity, smoking, and educational level showed inconsistent relations with waist girth. The prevalence of year 15 coronary calcification increased progressively across rising waist girth tertiles (P < 0.001).

The associations of baseline waist girth and WHR with the year 15 presence of CAC are shown in Table 2. After adjustment for baseline age, sex, race, and clinical center, there was a direct association between baseline waist girth and year 15 CAC and between baseline WHR and year 15 CAC (P for trend < 0.001 for both). Associations persisted after additional adjustment for baseline physical activity, cigarette smoking, educational level, and alcohol intake. For subjects in the highest tertile of baseline waist girth (≥84.3 cm and ≥75.5 cm in men and women, respectively) versus subjects in the lowest tertile, CAC risk over 15 y was 1.9 (95% CI: 1.36, 2.65). We observed a similar association between baseline waist girth or WHR and CAC risk (P for trend = 0.01) after adjustment for multiple risk factors (model 2). For persons in the highest versus lowest tertile of WHR (≥0.85 and ≥0.75 in men and women, respectively), the odds ratio for CAC risk was 1.7 (95% CI: 1.23, 2.41). Associations persisted after additional adjustment for systolic blood pressure, fasting insulin, diabetes, and antihypertensive medication use. As Table 2 shows, the association between waist girth and CAC risk was significant (P for trend = 0.01) after adjustment for these factors (model 3). A similar association was observed between baseline WHR and CAC risk (P for trend = 0.03). However, the associations were attenuated and became nonsignificant after additional adjustment for blood lipids (HDL and total cholesterol and triacylglycerol) (model 4). Further adjustment for the ratio of total to HDL cholesterol did not alter the association of waist girth or WHR to CAC (data not shown).

We also examined the associations of year 10 waist girth and WHR and year 15 waist girth with the presence of CAC at year 15. Substantial concordance of these findings with those using baseline waist girth or WHR as the exposure variable was expected, given the relatively high race- and sex-adjusted correlations of these variables with each other and over time (Table 3). The odds of having CAC, after adjustment for age, sex, race, clinical center, physical activity, cigarette smoking, educational level, and alcohol intake (adjustments analogous to those of model 2 in Table 2), in subjects in the highest tertile of waist girth at year 10 and year 15 were 1.7 (95% CI: 0.73, 1.47) and 1.8 (95% CI: 1.28, 2.47) times, respectively, the odds in subjects in the lowest waist girth category, as shown in Figure 1. Also shown in Figure 1 is that subjects in the highest tertile of WHR at year 10 had odds of year 15 CAC 1.8 times (95% CI: 1.25, 2.51) those of subjects in the lowest tertile of WHR at year 10.

Several supplemental analyses were run. First, further analyses studied the association of change in waist girth or WHR with year 15 CAC. Changes in waist girth (from baseline to year 15) and WHR (from baseline to year 10) were unrelated to year 15 CAC, whether in models that included the corresponding baseline measure or in those that excluded it. Second, we assessed a possible race × sex interaction for baseline waist girth in predicting year 15 CAC in model 2; the P value for the race × sex interaction was 0.37. Significant trends toward increasing year 15 CAC across baseline waist girth or WHR categories were seen in models stratified by sex or by race. Third, we examined the
associations categorizing sex-specific waist girth quintiles at baseline and found that the ORs (95% CI) compared with the smallest waist girth quintile (quintile 1) in model 2 were 1.06 (0.59, 1.88) for quintile 2, 1.21 (0.68, 2.14) for quintile 3, 1.50 (0.86, 2.62) for quintile 4, and 2.52 (1.47, 4.32) for quintile 5 ($P_{\text{for trend}}$ < 0.001). Analysis of baseline WHR in sex-specific quintiles yielded a similar association in model 2: 1.00 (lowest quintile), 1.08 (0.66, 1.74), 0.94 (0.58, 1.54), 1.54 (0.98, 2.42), and 1.99 (1.28, 3.08) ($P_{\text{for trend}}$ = 0.001). Fourth, we also adjusted for variables in model 1 plus physical activity, cigarette smoking, education, and alcohol intake. Fifth, we found that associations of year 15 CAC with BMI were similar to those reported here for baseline waist girth or WHR, but each fitness variable became nonsignificant when any 2 of those variables were in the model simultaneously (data not shown).

### DISCUSSION

Although abdominal obesity is considered a risk factor for CVD incidence and mortality (21–25), little research has been conducted on the association between abdominal obesity and early atherosclerosis. Our major finding was that abdominal obesity measured by waist girth or WHR is directly associated with a higher prevalence of CAC over a period of 15 y. Persons in the highest tertiles of baseline waist girth and WHR had year 15 CAC odds, after adjustment for age, sex, race, center, physical activity, cigarette smoking, educational level, and alcohol intake, 1.9 times (95% CI: 1.54, 3.79) those of men and women with smaller waist girth at baseline in model 2.

### TABLE 2

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>$P_{\text{for trend}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist girth (n)</td>
<td>277</td>
<td>62</td>
<td>79</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td>Adjusted for age, sex, race, and clinical center (model 1)</td>
<td></td>
<td>1.00</td>
<td>1.08 (0.76, 1.54)</td>
<td>1.86 (1.33, 2.58)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Multivariate models</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td>1.00</td>
<td>1.17 (0.82, 1.68)</td>
<td>1.90 (1.36, 2.65)</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Model 3</td>
<td>1.00</td>
<td>1.12 (0.78, 1.62)</td>
<td>1.61 (1.12, 2.31)</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Model 4</td>
<td>1.00</td>
<td>1.03 (0.71, 1.49)</td>
<td>1.21 (0.83, 1.77)</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>WHR (n)</td>
<td>277</td>
<td>62</td>
<td>77</td>
<td>138</td>
<td></td>
</tr>
<tr>
<td>Adjusted for age, sex, race, and clinical center (model 1)</td>
<td></td>
<td>1.00</td>
<td>1.08 (0.76, 1.55)</td>
<td>1.92 (1.38, 2.68)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Multivariate models</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td>1.00</td>
<td>1.03 (0.72, 1.48)</td>
<td>1.72 (1.23, 2.41)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Model 3</td>
<td>1.00</td>
<td>0.98 (0.68, 1.42)</td>
<td>1.47 (1.03, 2.09)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Model 4</td>
<td>1.00</td>
<td>0.89 (0.61, 1.29)</td>
<td>1.19 (0.83, 1.72)</td>
<td>0.32</td>
<td></td>
</tr>
</tbody>
</table>

1 $n = 2951$ for both measurements. CARDIA, Coronary Artery Risk Development in Young Adults.
2 Tertile cutoffs are <77.5, 77.5–84.3, and ≥84.3 cm (men) and <68, 68–75.5, and ≥75.5 cm (women) for waist girth and <0.81, 0.81–0.85, and ≥0.85 (men) and <0.71, 0.71–0.75, and ≥0.75 (women) for WHR.
3 Adjusted for variables in model 1 plus physical activity, cigarette smoking, education, and alcohol intake.
4 Adjusted for variables in model 2 plus systolic blood pressure, fasting insulin concentrations, diabetes, and antihypertensive medication use.
5 Adjusted for variables in model 3 plus blood lipids (HDL- and total cholesterol and triacylglycerol concentrations).

### TABLE 3

<table>
<thead>
<tr>
<th>Variables</th>
<th>Waist girth</th>
<th>WHR</th>
<th>BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Baseline</td>
<td>Baseline</td>
<td>Baseline</td>
</tr>
<tr>
<td>Year 10</td>
<td>Baseline</td>
<td>Year 10</td>
<td>Year 10</td>
</tr>
<tr>
<td>Year 15</td>
<td>Baseline</td>
<td>Year 10</td>
<td>Year 15</td>
</tr>
</tbody>
</table>

1 CARDIA, Coronary Artery Risk Development in Young Adults.
2 $P < 0.01$. 

Our findings were similar after further adjustment for baseline blood pressure, insulin, diabetes, and antihypertensive medication use (P for trend = 0.01) but were further attenuated to nonsignificance after additional adjustment for blood lipids. Thus, of the factors that could well be in the causal pathway between adiposity and CAC presence, blood lipids appeared to be the ones that explained the mechanisms underlying the association between adiposity and the presence of CAC.

In our study, a dose-response relation between abdominal obesity and the presence of CAC persisted across baseline, year 10, and year 15 examinations. Abdominal obesity measured in 1985–1986, 1995–1996, and 2000–2001 was positively associated with the odds of having CAC in 2000–2001. Our findings suggest that waist girth and WHR are risk factors for early atherosclerosis, which is consistent with the National Institutes of Health guidelines (20).

Several studies have reported that abdominal obesity is associated with metabolic risk factors and CVD mortality (21–25, 28, 29). In our data, waist girth is directly associated with systolic blood pressure, blood lipids, and blood concentrations of glucose and insulin. Although we found that blood lipids were the factors that statistically explained the association of abdominal adiposity and the presence of CAC, elevated blood lipids have complex relations with blood pressure, blood glucose, and insulin. It is plausible that greater abdominal obesity may enhance atherosclerosis through disturbances in any of these variables. Greater visceral fat is associated with insulin resistance and hyperinsulinemia, and that association contributes to the progression of atherosclerosis (10). Greater visceral fat also is associated with perturbations of the hemostatic and coagulation systems. For instance, greater visceral fat elevates the release of plasminogen activator inhibitor-1 (PAI-1), increases the markers of hypercoagulability and hypofibrinolysis, and disturbs PAI-1 activity, which impairs the fibrinolytic system and contributes to thrombotic vascular disease in the artery (30, 31). Abdominal obesity is positively associated with LDL cholesterol and oxidized LDL, and that association contributes to endothelial cell injury and subsequent thrombus formation (1, 2). In general, abdominal obesity can play a prothrombotic role by increasing plasma lipid and lipoprotein concentrations (28, 29) and inflammatory markers [e.g., C-reactive protein, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6); 32–34] and by elevating blood viscosity (35) and impairing fibrinolysis (36), all of which may contribute to atherosclerotic vascular disease.

A strength of this study is that our data represent population-based samples of US African American and white young men and women. To our knowledge, this is the first study to investigate the relation of abdominal obesity measured by waist girth or WHR to the presence of CAC in young adults. Further studies are needed to determine whether abdominal obesity is associated with presence of CAC across different ethnic groups or in diabetic or hypertensive persons. One limitation of our study is that we did not assess CAC before year 15, and thus we cannot tell whether the presence of CAC is incident or prevalent. However,
other studies found that CAC was present in higher proportions in the older samples (eg, The Multi-Ethnic Study of Atherosclerosis Study), and it is likely that much of the CAC seen in the CARDIA Study developed between baseline (average age: 25 y) and year 15. Limitations of our study also include the relatively small numbers of persons with CAC, which restricted our power to examine subgroups. Furthermore, it is known that CT images of the heart are noisier and more difficult to interpret in fatter than in thinner persons. However, whereas conservative reading in the face of greater noise could explain the lack of relation cross-sectionally between BMI and CAC presence (9), both concurrent and baseline waist girth were related to the presence of CAC. Another possible limitation is that some, but not all, subjects weighing >360 lb were excluded because the CT table could not hold a person of that weight, which may have created an unknown bias. Finally, adjustment for BMI attenuated the association of waist girth with CAC presence. Whereas BMI is a measure of total-body fatness and it seems that waist girth would be a measure of fatness more specific to the abdominal region, Table 3 shows the very high correlation between waist girth and BMI. Apparently BMI carries a considerable amount of information about fatness in the abdomen; we interpret the attenuation as partially adjusting waist girth for itself.

In conclusion, we found that abdominal obesity measured by waist girth or WHR is associated with early coronary calcification in young adults. Our study adds to the body of knowledge suggesting that waist girth in young adulthood may help to identify persons with potential future CHD risk that is most likely due to associated lipid and other metabolic abnormalities.

The authors thank the staff of and participants in the CARDIA Study for their important contributions.

The authors’ responsibilities were as follows—CDL and DRJ: proposed the study, analyzed the data, and wrote the manuscript; and PJM, CI, and AH: assisted with statistical analyses and revision of the manuscript. None of the authors had a personal or financial conflict of interest.

REFERENCES


Sweet taste preferences are partly genetically determined: identification of a trait locus on chromosome 16

Kaisu Keskitalo, Antti Knaapila, Mikko Kallela, Aarno Palottie, Maija Wessman, Sampo Sammalisto, Leena Peltonen, Hely Tuorila, and Markus Perola

ABSTRACT

Background: Humans have an innate preference for sweet taste, but the degree of liking for sweet foods varies individually.

Objective: The proportion of inherited sweet taste preference was studied. A genome-wide linkage analysis was performed to locate the underlying genetic elements in the genome.

Design: A total of 146 subjects (32% men, 68% women) aged 18–78 y from 26 Finnish families evaluated the intensity and pleasantness of 3 supratreshold solutions of sucrose (3.0%, 7.5%, and 18.75%) and plain water and the intensity of filter paper impregnated with 6-n-propylthiouracil (PROP). The subjects also reported the pleasantness and the use frequency of 5 sweet foods (chocolate, candy, ice cream, sweet desserts, and sweet pastry) and completed a food-behavior questionnaire that measured their craving for sweet foods.

Results: Of the chemosensory functions, the pleasantness rating of the strongest (18.75%) sucrose solution and the intensity rating of PROP yielded the highest heritability estimates (41% and 66%, respectively). The pleasantness and the use frequency of sweet foods (both variables calculated as a mean of ratings for 5 food items) and the craving for sweet foods showed significant heritability (40%, 50%, and 31%, respectively). A logarithm of odds score of 3.5 ($P = 0.00003$) was detected for use frequency of sweet foods on chromosome 16p11.2 (marker D16S753).

Conclusions:Sweet taste preferences are partly inherited. Chromosome 16p11.2 may harbor genetic variations that affect the consumption of sweet foods.

KEY WORDS Family study, food preferences, genetic linkage, heritability, human genetics, sweet taste

INTRODUCTION

Humans are genetically predisposed to prefer sweet taste. Because sweet foods are naturally good and are safe sources of energy and nutrients, adaptive evolutionary development has resulted in a preference for them (1). However, this evolution happened long ago when food was scarce. Today, with a great variety of sweet foods readily available in Western countries, the preference for these foods may also have disadvantages.

The perception of sweet taste is initiated by the interaction of a tastant with a TAS1R2/TAS1R3 heterodimer (taste receptor type 1, members 2 and 3)—a G protein–coupled receptor (GPCR) localized in the taste buds of the tongue and the palate (2, 3). The human sweet taste receptor genes, TAS1R2 and TAS1R3, which encode the receptors reacting with sweet tastants, are both located on chromosome 1p36 (4). These genes were first discovered in mice. Numerous groups have since investigated the effect of polymorphisms or knockout of the Tas1r3 gene (Sac locus) on sweet taste sensitivity and preferences in mice. Although several studies have shown that sequence variations in the Tas1r3 gene affect sweetener preferences of inbred mice strains (5, 6), Sclafani (7) found no differences in motivation to obtain sugar between “low-sweetener-preferring” (129P3/J) and “high-sweetener-preferring” (C57BL/6J) strains. In addition, despite Tas1r3 knockout mice having diminished behavioral and neural responses to sugars (8, 9), no differences in sucrose detection thresholds between wild-type and knockout strains were observed by Delay et al (10). Thus, the effect of variations in Tas1r3 on the behavior of mice remains unclear.

Humans differ in their liking for sweet foods (11). Moreover, environmental factors play an important role in the development of preferences for sweet foods and other foods (for a review, see 12). Although newborns prefer sugar solutions to water (13, 14), dietary experiences modify the degree of the preference for sweet taste already at the age of 6 mo (15). To our knowledge, no studies of how quantitative trait loci (QTL) affect sweet taste preferences in humans have been published to date. Earlier genome-wide linkage studies have concentrated on identifying regions harboring genes affecting macronutrient intake. Collaku et al (16) found evidence of significant and suggestive linkage on chromosomes 16p11.2 and 6p21.3.

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2 Supported by the Academy of Finland (206327, 200923, and 00213), the GenomeEUtwin Project (QLG2-CT-2002-01254), EuroHead (LSHM-CT-2004-504837), the National Institutes of Health (RO1 NS37675), the Finnish Heart Association, the Oxnard Foundation, the Finnish Neurology Foundation, the Helsinki University Central Hospital, and the Sigrid Juselius Foundation.

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1p21.2 and 20q13.3 for total energy intake and on 12q14.1 for fat intake, and Cai et al (17) found evidence of significant and suggestive linkage on chromosome 2p22 for saturated fat intake [logarithm of odds (LOD) 2.62]. Our aim was to determine the proportion of the genetic factors contributing to the sweetness perception of aqueous solutions and sweet food preferences in a population randomly selected in terms of sweet taste preference-related phenotypes. If a trait was found to be heritable, we further aimed to identify the corresponding loci in the genome using genome-wide marker data in families.

SUBJECTS AND METHODS

Subjects

The sample consisted of 146 adult members (46 males, 100 females) from 26 Finnish families, including 9 spousal, 63 parent-offspring, 137 sibling, 4 half-sibling, 127 avuncular, and 37 first cousin pairs. The subjects were participating in a migraine study, either as migraine patients (83.6%) or as healthy family members (16.4%) (18). The subjects, randomly selected in terms of taste phenotypes, were 18–78 y of age and had a mean (±SD) age of 49.0 ± 14.8 y. The height and weight of the subjects were measured at the clinic visit by a nurse. Body mass index (in kg/m²) ranged from 15.3 to 42.0 (± SD: 26.0 ± 4.6). The study protocol was approved by the Ethical Committee of Helsinki University Central Hospital. All participants gave informed consent.

Sample preparation

Sensitivity to 6-n-propylthiouracil (PROP) was screened by using the filter paper method (19). The PROP test was included in the protocol because both the threshold and the suprathreshold intensity of PROP are known to be inherited (20, 21); the filter paper test was expected to provide a rough estimate of heritability. The finding of a significant heritability estimate for PROP intensity would support the appropriateness of the rating procedure for the investigation of heritable effects on taste perception. The PROP filter papers were prepared by soaking filter paper disks (Whatman 1) in saturated PROP (6-propyl-2-thiouracil; Sigma-Aldrich Chemie GmbH 82460, Steinheim, Germany) water solution at boiling temperature for 30 s. The disks were left to dry overnight on aluminum foil at room temperature or in an oven at 121 °C for 1 h. The disks were weighed before and after the procedure, and the amount of absorbed PROP was calculated as the difference in weight. The filter paper disks were cut into square-shaped pieces so that each contained 0.6 mg PROP (1.2 cm²). The PROP-containing filter paper pieces and plain filter paper pieces were stored in sealed plastic sachets at room temperature for a maximum of 3 mo before use.

Three suprathreshold sucrose (Danisco Sugar, Kantvik, Finland) solutions (3.0%, 7.5%, and 18.75% wt:vol) plus plain water were prepared in tap water. The samples were stored in the refrigerator (7 °C) overnight and were then brought to room temperature. A separate series of 3 sodium chloride solutions (0.2%, 0.5%, and 1.25% wt:vol) plus plain water was also prepared and presented to the subjects.

Chemosensory tests

The subjects visited the clinic after fasting overnight (12 h). Before evaluating the intensity of the PROP filter paper, the subjects were exposed to pure filter paper to later be able to distinguish the taste of the paper from that of PROP. The subjects placed filter paper containing PROP into the mouth, kept it on the tongue for =10 s, and after waiting a short while (the strongest sensation of PROP often comes with a delay) rated the intensity using a vertical 12.0-cm labeled magnitude scale (22).

The sweet and salty samples plus plain water as a control in both series (15 mL each) were labeled with 3-digit random codes. The order of sweet and salty series and the order of 4 samples within each series were randomized. The subjects were requested to rinse their mouths with tap water before starting the evaluations and between samples. The samples and the rinsing water were served at room temperature. The subjects were instructed to take the whole 15 mL sample into their mouth, twirl it around, expectorate, and provide a rating by placing a vertical line on each scale.

The instructions were given both orally and in written form, and the test administrator was present throughout the testing procedure. The intensity and pleasantness of the sweet or salty taste in the solutions were evaluated by using a 12.5-cm horizontal labeled magnitude scale (22) and labeled affective magnitude scale (23), respectively. The distance of the hash mark from the left end of the line made by the subject was measured manually. The verbal labels and their positions (cm from the left end) on the line were for the following intensity ratings: “no taste” (0.0), “barely detectable” (0.2), “weak” (0.7), “moderate” (2.1), “strong” (4.4), “very strong” (6.7), and “strongest imaginable sensation” (12.5). On the pleasantness scale, the labels were “greatest imaginable unpleasantness” (0.0), “extremely unpleasant” (1.3), “very unpleasant” (2.2), “moderately unpleasant” (4.0), “slightly unpleasant” (5.5), “neither pleasant nor unpleasant” (6.2), “slightly pleasant” (6.8), “moderately pleasant” (8.4), “very pleasant” (9.2), “extremely pleasant” (11.0), and “greatest imaginable pleasantness” (12.5). Because of the lack of the word dislike in the Finnish language, the translations refer to the pleasantness of the perception rather than to liking. In addition, the subjects evaluated how hungry they felt using a 9-point category scale (1 = not hungry at all; 9 = very hungry).

Questionnaire data

The subject rated the pleasantness and the use frequency of 30 foods using 7 categories. The response alternatives for pleasantness were 1 = very unpleasant, 2 = fairly unpleasant, 3 = slightly unpleasant, 4 = neither pleasant nor unpleasant, 5 = slightly pleasant, 6 = fairly pleasant, 7 = very pleasant. The response alternatives for use-frequency 1 = never, 2 = once a month or less often, 3 = 1–2 times a month, 4 = once a week, 5 = a couple of times a week, 6 = almost every day, and 7 = at least once a day. For further analysis, foods were categorized by using principal component analysis and reliability analysis, and a group of sweet foods, including 5 food items with sweetness as the salient attribute (chocolate, sweets, ice cream, sweet pastry, and sweet desserts), was identified. Because the sweet foods formed a minor part of the questionnaire, the subjects were unaware of our particular interest in them. In addition to sweet foods, clusters of salty, fatty, and snack foods were identified. From here on we focus on the cluster of sweet foods only. The results for the pleasantness and use-frequency ratings of the other food groups are available from the authors on request. The phenotypes for pleasantness and use frequency of sweet foods were...
The intensity of sucrose solutions was measured using a 7-point scale, with values ranging from 0 to 12.5. The perceived intensities of sucrose samples were observed, and the genetic distance between markers was interpreted using DeCode map as a backbone to our in-house program Cartographer (27).

PedCheck (28) was used to check the genotype data for Mendelian inconsistencies. No level 0, 1, or 2 errors were detected by PedCheck. In addition, MERLIN (29) was used to screen for unlikely but Mendelian-consistent genotypes. The unlikely genotypes detected by the MERLIN error detection algorithm were erased from the pedigree file using the program Pedwipe provided by MERLIN (29).

### Statistical analysis

Singlepoint and multipoint linkage analyses were performed by using a variance component method implemented in MERLIN (29) to locate genetic elements underlying traits analyzed across the genome. In the variance components framework, the expected allele sharing at a putative quantitative trait locus is correlated with their phenotypic covariance, thus evaluating the linkage between a certain genetic marker and the trait of interest. Usually, a LOD score of 3 ($P$ value of a single test $=0.0001$) is regarded as significant for a monogenic trait and implies that the genetic marker is close to the trait locus, i.e., the 2 loci are linked. For a complex trait, the concept of a significant LOD score is somewhat more ambiguous (30). In addition to linkage evidence, the variance component method also provides a heritability estimate for the trait analyzed. This estimate expresses the proportion of the variation that makes family members more similar with each other, including the effects of both their shared genetic parameters and their common environment.

The age, sex, and migraine status of each subject at the time of the clinic visit were used as covariates in the quantitative genetic analysis. The self-rated hunger was used as covariate in the quantitative genetic analysis. In the linkage analysis, only the significant covariates were included [significances obtained from the heritability analysis of program SOLAR (31) assuming a polygenic model]. Except for quantitative genetic modeling, all statistical analyses were carried out by using the SPSS statistical package (32).

### RESULTS

Characteristics and heritability estimates of the traits are shown in Table 1 and Table 2. Although differences in the mean perceived intensities of sucrose samples were observed, the mean pleasantness ratings of the samples did not differ. However, there was a trend for an increasing SD with increasing sweetness intensity in both the intensity and pleasantness evaluations. The 2 subjects who had given very low (<2 cm) intensity

### TABLE 1

Ratings and heritability estimates for the various traits

<table>
<thead>
<tr>
<th>Trait</th>
<th>Theoretical range</th>
<th>$\bar{x} \pm SD$</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Heritability estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity of sucrose solutions</td>
<td>0–12.5</td>
<td>0.4 ± 0.6</td>
<td>0.0</td>
<td>5.3</td>
<td>7.0</td>
</tr>
<tr>
<td>3.0%</td>
<td>0–12.5</td>
<td>1.7 ± 1.2</td>
<td>0.0</td>
<td>7.0</td>
<td>0.0</td>
</tr>
<tr>
<td>7.5%</td>
<td>0–12.5</td>
<td>3.7 ± 2.0</td>
<td>0.0</td>
<td>11.0</td>
<td>0.0</td>
</tr>
<tr>
<td>18.75%</td>
<td>0–12.5</td>
<td>5.3 ± 2.3</td>
<td>0.9</td>
<td>11.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Pleasantness of sucrose solutions</td>
<td>0–12.5</td>
<td>6.7 ± 1.4</td>
<td>1.8</td>
<td>11.1</td>
<td>27.3</td>
</tr>
<tr>
<td>3.0%</td>
<td>0–12.5</td>
<td>6.7 ± 1.3</td>
<td>2.4</td>
<td>10.4</td>
<td>19.5</td>
</tr>
<tr>
<td>7.5%</td>
<td>0–12.5</td>
<td>6.7 ± 1.7</td>
<td>2.4</td>
<td>9.9</td>
<td>29.2</td>
</tr>
<tr>
<td>18.75%</td>
<td>0–12.5</td>
<td>6.2 ± 2.0</td>
<td>1.3</td>
<td>10.1</td>
<td>40.9</td>
</tr>
<tr>
<td>Intensity of 6-n-propylthiouracil</td>
<td>0–12.0</td>
<td>3.9 ± 2.6</td>
<td>0.0</td>
<td>12.0</td>
<td>65.5</td>
</tr>
<tr>
<td>Pleasantness of sweet foods</td>
<td>1–7</td>
<td>5.6 ± 1.0</td>
<td>2.0</td>
<td>7.0</td>
<td>40.3</td>
</tr>
<tr>
<td>Use frequency of sweet foods</td>
<td>1–7</td>
<td>3.5 ± 0.8</td>
<td>1.4</td>
<td>5.2</td>
<td>50.2</td>
</tr>
<tr>
<td>Craving for sweet foods</td>
<td>1–7</td>
<td>3.8 ± 1.6</td>
<td>1.0</td>
<td>6.8</td>
<td>31.0</td>
</tr>
</tbody>
</table>

*Age, sex, and migraine status were used as covariates for all of the traits and self-rated hunger for the chemosensory measurements.*
ratings for the 18.75% sucrose solution had rated the intensities of the sweet solutions in an ascending order and had given higher intensity ratings for the salty solutions. Thus, we concluded that these subjects were not ageusic; therefore, they were not excluded from the analyses. The hunger estimates varied from 1 to 9; the mean (± SD) was 4.9 ± 2.2.

Our study design allowed evaluation of the effects of age, sex, self-rated hunger, and migraine status on the traits. The pleasantness rating of the solution containing 0.2% NaCl is clearly understood, and the scale was not the reason for the low heritability estimates of the pleasantness ratings of the 2 strongest sucrose solutions of 7.5% and 18.75% (29.2% and 40.9%, respectively) and that of the plain water (27.3%) were all significant. The heritability estimates for the pleasantness evaluation and the user frequency of sweet foods were 40.3% and 50.2%, respectively. The heritability of the score on the Craving for Sweet Foods scale was lower, 31.0%. The heritability estimates of the intensity and pleasantness ratings of the salty solutions were all very low. The only significant heritability estimates were obtained for the pleasantness rating of the 0.2% NaCl solution and that of pure water (33.1% and 24.3%, respectively). The salty taste of the solution containing 0.2% NaCl is clearly detectable, albeit mild (33).

Pearson’s correlation coefficients between sweet taste perception and preference-related traits are provided in Table 3. Several significant correlations among the measured traits suggest that a common factor underlies them. The sweet taste preference-related phenotypes did not correlate significantly with PROP intensity (r < 0.08) or BMI (r < 0.15).

### Variance component quantitative trait linkage analysis

Quantitative trait linkage analysis for the use frequency of sweet foods produced a multipoint LOD score of 3.5 on chromosome 16p11.2 (Table 4), which peaked at the marker 0.0143 (Table 2).

### Table 2

<table>
<thead>
<tr>
<th>Trait</th>
<th>Rating</th>
<th>Heritability estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity of salty solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>0.4 ± 0.5</td>
<td>4.6</td>
</tr>
<tr>
<td>0.2%</td>
<td>1.7 ± 1.5</td>
<td>4.4</td>
</tr>
<tr>
<td>0.5%</td>
<td>3.6 ± 2.1</td>
<td>0.0</td>
</tr>
<tr>
<td>1.25%</td>
<td>5.8 ± 2.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Pleasants of salty solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>6.8 ± 1.5</td>
<td>24.3</td>
</tr>
<tr>
<td>0.2%</td>
<td>5.8 ± 1.4</td>
<td>33.1</td>
</tr>
<tr>
<td>0.5%</td>
<td>5.3 ± 1.6</td>
<td>0.0</td>
</tr>
<tr>
<td>1.25%</td>
<td>4.2 ± 1.8</td>
<td>0.0</td>
</tr>
</tbody>
</table>

1. Age, sex, migraine status, and self-rated hunger were used as covariates in the models.
2. All values are x ± SD.

We found a correlation with age for only the score on the Craving for Sweet Foods scale (r = −0.37, P < 0.001). The negative correlation means that younger subjects have a greater tendency than do older subjects to crave sweet foods. Self-rated hunger correlated significantly with the intensity ratings of 3.0% (r = 0.17, P = 0.040), 7.5% (r = 0.24, P = 0.004), and 18.75% (r = 0.23, P = 0.005) sucrose solutions. A negative correlation was found between the ratings of hunger and pleasantness for the 7.5% sucrose solution (r = −0.23, P = 0.006).

The heritability estimates for the intensity evaluations of sweet samples were all near zero. The heritability of the intensity evaluation of PROP filter paper was, however, high (66%). This implies that the labeled magnitude scale (LMS) was properly understood, and the scale was not the reason for the low heritability estimates in the sweetness intensity evaluations. The heritability estimates of the pleasantness ratings of the 2 strongest sucrose solutions of 7.5% and 18.75% (29.2% and 40.9%, respectively) and that of the plain water (27.3%) were all significant. The heritability estimates for the pleasantness evaluation and the user frequency of sweet foods were 40.3% and 50.2%, respectively. The heritability of the score on the Craving for Sweet Foods scale was lower, 31.0%. The heritability estimates of the intensity and pleasantness ratings of the salty solutions were all very low. The only significant heritability estimates were obtained for the pleasantness rating of the 0.2% NaCl solution and that of pure water (33.1% and 24.3%, respectively). The salty taste of the solution containing 0.2% NaCl is clearly detectable, albeit mild (33).

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>Intensity of sucrose solution</th>
<th>Pleasantness of sucrose solution</th>
<th>Sweet foods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
<td>3.0%</td>
<td>7.5%</td>
</tr>
<tr>
<td>Intensity of sucrose solution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0%</td>
<td>0.12</td>
<td>0.29f</td>
<td></td>
</tr>
<tr>
<td>7.5%</td>
<td>-0.03</td>
<td>-0.23f</td>
<td>-0.31f</td>
</tr>
<tr>
<td>18.75%</td>
<td>-0.03</td>
<td>-0.24f</td>
<td>-0.23f</td>
</tr>
<tr>
<td>Pleasantness of sucrose solution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweet foods</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pleasants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Use</td>
<td>-0.04</td>
<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Craving</td>
<td>-0.01</td>
<td>0.05</td>
<td>-0.07</td>
</tr>
</tbody>
</table>

1. P < 0.05.
2. P < 0.01.
D16S753. The single point LOD score at this marker was 2.9. The information content on the peak area was fairly high (78%), and a decrease of one LOD (34) on this locus covers a region of ≈10 cM harboring >30 genes. However, no obvious candidate genes for this trait were identified. A significant LOD for this novel phenotype and low (\( r = 0.06 \)) spousal correlation suggest that the heritability estimate here indicates a true effect of genes rather than a mere familial correlation. In addition, some evidence for linkage for the use frequency of sweet foods was found on chromosomes 9q32.1 (LOD = 2.1 marker D9S286), 20q13.2 (LOD = 1.9 marker D20S480), and 3p26.3 (LOD = 1.9 marker D3S2387). A graph of the genome-wide multipoint linkage scan with the information contents of the markers for the use frequency of sweet foods is presented in Figure 1. The corresponding multipoint and single point linkage scans of chromosome 16 are shown in Figure 2.

The multipoint linkage analysis also produced a LOD score of 1.9 on chromosome 1q41 for the pleasantness rating of 18.75% sucrose solution (single point LOD score at the marker = 0.68). The genome-wide multipoint scan results are shown in Figure 3, and the multipoint and single point scans of chromosome 1 are shown in Figure 4.

**Genome-wide P values**

To determine the empirical significance of our linkage findings, we simulated 100 genome-wide scans of comparable structure using MERLIN and analyzed each simulated scan identically to the original data analysis. MERLIN performs gene-dropping simulation while retaining the genetic map, phenotype data, pedigree structure, and missing genotype data patterns, creating comparable data with random marker genotypes. Because the data are simulated under the hypothesis of no linkage, any linkage seen is due to chance alone, which therefore allows the evaluation of the false-positive rate of the data set analyzed. The empirical \( P \) value for a LOD score was defined as the proportion of simulated genomes where the LOD score in question was reached or exceeded. Subsequently, the corresponding 95% Wilson CIs were calculated for the empirical \( P \) value (35). The

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<table>
<thead>
<tr>
<th>Trait and covariate</th>
<th>LOD</th>
<th>Marker</th>
<th>Location</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Use frequency of sweet foods</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine, age</td>
<td>3.5</td>
<td>D16S753</td>
<td>16p11.2</td>
<td>0.00003</td>
</tr>
<tr>
<td>Migraine, age</td>
<td>2.0</td>
<td>D9S286</td>
<td>9q32.1</td>
<td>0.0010</td>
</tr>
<tr>
<td>Migraine, age</td>
<td>1.9</td>
<td>D3S2387</td>
<td>3p26.3</td>
<td>0.0014</td>
</tr>
<tr>
<td>Migraine, age</td>
<td>1.9</td>
<td>D20S480</td>
<td>20q13.2</td>
<td>0.0015</td>
</tr>
<tr>
<td>Pleasantness of 18.75% sucrose solution</td>
<td>1.9</td>
<td>D1S549</td>
<td>1q41</td>
<td>0.0014</td>
</tr>
</tbody>
</table>

\( ^1 \) LOD, logarithm of odds.

---

**FIGURE 1.** Genome-wide multipoint linkage analysis results (thick line) and the information content curves (thin line) for the use frequency of sweet foods. LOD, logarithm of odds.
best LOD score (3.5 on chromosome 16p11.3) produced an empirical $P$ value of 0.07 (95% Wilson CI: 0.03, 0.14) in the permutation analyses. The other suggestive loci did not survive the permutation tests, the second lowest empirical $P$ value being 0.25 (95% Wilson CI: 0.18, 0.34) for the LOD score of 1.9 for pleasantness rating of 18.75% sucrose solution.

**DISCUSSION**

We found evidence of significant linkage between the use frequency of sweet foods and a marker located on chromosome 16. Our results show that pleasantness of an extremely sweet solution and pleasantness and use frequency of sweet foods are
partly heritable; 40–50% of the variation in these traits is explained by inherited mechanisms. The intensity and pleasantness ratings of the salty solutions were, in turn, mostly not inherited. The latter observation agrees with earlier results. A comparison of our results with variance components obtained from twin studies suggests that the significant heritability estimates may be due to common family environment rather than to genetic effects (36).

To our knowledge, no other QTL affecting sweet taste preference in humans have been identified to date. Collaku et al (16) and Cai et al (17) have both performed genome-wide linkage analysis on macronutrient intakes, calculated from food-frequency-questionnaire data. Neither of the studies showed suggestive or significant linkages for sucrose intake, maybe because of the fairly general level of measurement of food intake. Studies evaluating the proportion of heritable effects on sweet taste preferences, with actual psychophysical testing of the subjects, are also very rare. Using data from 13 monozygotic and 10 dizygotic twin pairs, Krondl et al (37) found no significant heritability for the recognition threshold of sucrose or for preferences for and use frequency of 4 sweet foods (honey, jam, ice cream, and doughnuts) using the Holzinger index of heritability:

\[
\text{Holzinger index of heritability} = \frac{\text{Var}_{\text{MZ}} - \text{Var}_{\text{DZ}}}{\text{Var}_{\text{DZ}}}
\]

where Var is the within-pair variance of the mean difference. The negative result may have resulted because of the small sample size or because the statistical method used was not sufficiently sophisticated to reveal heritable effects.

The linkage peak for use frequency of sweet foods with a multipoint LOD score of 3.5 (empirical \(P = 0.07\)) was located on chromosome 16p11.2. This area does not harbor genes known to affect the trait. However, Chr16p11.2 does contain 3 locations of hypothetical proteins, ie, locations harboring a gene whose function remains unknown. The \(p\) arm of chromosome 16 was previously linked to taste-related traits. Drayna et al (38) identified a QTL on Chr16p on 2-locus whole-genome scan conditional on Chr7 QTL for PTC (phenylthiocarbamide) tasting ability. The QTL on Chr16p provided a 2-locus LOD score of 3.33 at 14 cM. However, though located in the same chromosomal arm as our peak for the use frequency of sweet foods (marker located at 56.8 cM), these peaks are rather far away from each others. Another interesting linkage was obtained for the pleasantness of the 18.75% sucrose solution. Although the phenotype may better reflect the biological mechanism underlying the sweetness preference than a variable obtained from the use-frequency questionnaire, the result on chromosome 1q41 needs to be replicated in another sample because it did not reach genome-wide significance. Our linkage analysis of PROP intensity did not show any significant or suggestive QTLs. This discrepancy with earlier studies (20, 38) finding linkage on Chr7 (gene TAS2R38) may be due to methodologic differences in PROP sensitivity measurement.

The fact that a significant linkage result was found for use frequency, and not for the pleasantness ratings, does not disprove the hypothesis that the same genes affect these heritable traits. Many of the variables were correlated, which implies that a common factor underlies these traits. Evaluating the use frequency of a food is perhaps more exact than is evaluating pleasantness, because rating the pleasantness of foods without tasting or seeing them may target to different products (eg, a different type of candy) (39). Also, subjects may avoid using the ends of a hedonic scale (40). In the use-frequency evaluation, the central tendency is less likely, because both ends of the scale are explicit frequency estimations. Thus, the use-frequency evaluation may represent sweetness preference and thereby reveal the underlying genetic tendency to like (or dislike) sweetness. Measuring the
sweet taste preference in humans is complicated: intensity and pleasantness ratings of aqueous solutions may poorly generalize to behavior. On the other hand, sweet foods always possess sensory attributes other than sweetness, and the preferred level of sweetness is often food-specific (41). However, we decided to include many measures of sweet preferences because the predictive value of separate measures on actual dietary intake of sweet foods may be limited (42).

The use frequency may also be affected by many factors other than liking for foods. If people always ate foods that they like the best, we would expect the use frequency of sweet foods to be higher. Some factors affecting use frequency, apart from liking the food, may also be heritable. For example, 44% and 59% of the variation in cognitive restraint of eating was shown to be heritable in twin studies by de Castro (43) and Tholin et al (44), respectively.

Our results imply that the intensity perception of the supra-threshold sweet taste is not heritable. The affective processing of the sensation seems, however, to be partly genetically steered. Thus, it is not surprising that the LOD peak for use frequency of sweet foods was not located near the sweet taste receptors. Experiments with mice have shown that polymorphisms in sweet taste receptor genes do influence the preference for sweet solutions (5, 6). Whereas mice eat any acceptable and available food, the food choices in humans are more complex. For example, the brand knowledge has been suggested to alter the behavioral preferences and neural responses to a sweet, culturally familiar drink (45). No evidence of polymorphisms in taste receptor genes mediating the sweet taste preference in humans has been published. Different brain regions are responsive to sweetness intensity and pleasantness perceptions in humans (46), and one might therefore expect that different mechanisms underlie these phenotypes.

The men evaluated the 18.75% sucrose solution as more pleasant than did the women. This observation is consistent with the results of Conner and Booth (47). In their study of the most preferred concentration of sugar in a lime drink, the men showed a greater sweetness preference than did the females on average. A difference between migraine patients and healthy family members was found in the intensity evaluation of the weakly sweet 3.0% sucrose solution. Taste abnormality during an acute migraine attack has been reported by some migraine patients (48), but the degree of these patients’ taste abnormality when not having an attack has not been investigated. Significant, albeit not very high, correlations were found between self-rated hunger and intensity evaluations of all 3 sucrose solutions. This finding is in line with the literature. Caloric deprivation and hunger have been shown to increase taste sensitivity to sweet taste (49, 50). Age did not correlate with any of the psychophysical measurements. Although taste sensitivity decreases with age, the sensitivity to sweet taste does not decline as much as does the sensitivity to other tastes (51).

The strong correlations among heritable phenotypes, the limited sample size, and the inability to distinguish between effects of common family environment and genetic effects in our study call for further evaluation of the phenotypes and the genetic effects using larger populations of monzygous and dizygous twins or family members reared apart. Despite the limited sample size, the nature of variance component linkage analysis in which the possible phenotypic data errors also masquerade familial clustering, thus increasing noise and hindering signal detection, and the finding of both a significant LOD score (LOD = 3.5) and heritability estimate ($P = 0.007$) for a trait, do not support a false-positive result.

In conclusion, individual differences in sweet taste preferences appear to be partly heritable. A locus on chromosome 16 was found to affect the use frequency of sweet foods. This result can be considered to be very significant, because a sweet taste preference has not been previously shown to be heritable in humans. This observation broadens our understanding of human food choice.

We thank the families for participating in the study and Kaisu Taskila, Eija Hämäläinen, and Tanja Moilanen for excellent technical assistance.

The authors’ responsibilities were as follows—KK: drafted the manuscript; KK and AK: analyzed the data; MP and SS: assisted in the quantitative script; KK and AK: analyzed the data; MP and SS: assisted in the quantitative genetic analysis; MK, KK, AK, AP, and MW: collected the data; HT, LP, and MP: planned the study; MW: analyzed the genotypes. All authors contributed to the interpretation of the results and to the writing of the manuscript and accepted the final version. None of the authors had a conflict of interest.

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Predictors of serum ferritin and serum soluble transferrin receptor in newborns and their associations with iron status during the first 2 y of life\textsuperscript{1–3}

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ABSTRACT

Background: Adequate iron status at birth may prevent iron deficiency in early childhood.

Objectives: We aimed to identify predictors of serum ferritin (SF) and serum soluble transferrin receptor (sTfR) in healthy newborns and to relate these iron indexes to iron status in the first 2 y of life.

Design: By using bivariate correlations and linear regression, we related various factors in pregnancy to SF ($n = 363$) and sTfR ($n = 350$) in healthy, term infants. Measurements of cord SF and sTfR were compared with those of SF and sTfR at 6, 12, and 24 mo. All 4 measurements were available for 191 and 169 infants for SF and sTfR, respectively.

Results: Geometric mean (and 95% CI) cord SF and sTfR measurements were 159 (148, 171) $\mu$g/L and 7.3 (7.0, 7.6) mg/L, respectively. Cord SF correlated with sTfR ($r = -0.21, P < 0.001$). In regression analysis, cord SF correlated with smoking and the use of iron supplements during pregnancy (partial $r = -0.12$ and 0.16; $P < 0.05$ for both). Cord sTfR was associated with first trimester BMI, gestational age, and male sex (partial $r = 0.30, 0.24$, and 0.19, respectively; $P < 0.01$ for all). Cord SF correlated with SF at 6, 12, and 24 mo ($r = 0.45, 0.31$, and 0.16 respectively; $P < 0.05$ for all). At age 6 mo, 16 of 17 infants with SF <15 $\mu$g/L were boys.

Conclusions: Cessation of smoking and adequate iron prophylaxis during pregnancy may improve iron status in infancy. Cord SF is a predictor of iron status in the first 2 y of life. Boys are at particular risk of low iron status in early infancy. Am J Clin Nutr 2007;86:64–73.

KEY WORDS  
Iron status, iron deficiency, iron supplementation, serum ferritin, serum transferrin receptor, pregnancy, newborns, children, cord blood

INTRODUCTION

Adequate iron status is particularly important during pregnancy and during the child’s first 2 y of life to ensure the optimal development of the brain and nervous system. Iron deficiency anemia in this period may delay or impair the mental and physical development of children (1). School-aged children with low iron status have been shown to perform worse on standardized math tests than those with adequate iron status (2), and children with severe, chronic iron deficiency in infancy score lower in mental and motor functioning >10 y later, despite correction for iron deficiency (3). Studies in animal models have shown that iron deficiency affects brain development (4) and that prenatal and postnatal maternal iron deficiency is associated with behavioral effects in infant monkeys, even in the absence of iron deficiency (5). Studies of human infants indicate that iron deficiency may impair myelination in the central nervous system (6) and that the effects on transmission in the auditory and visual systems persist into childhood (7). A sufficient iron supply in pregnancy and the prevention of iron deficiency in infancy may therefore be of profound importance to the health and development of children.

Iron status usually is adequately assessed through the measurement of the concentrations of serum ferritin (SF) and serum soluble transferrin receptor (sTfR), which reflect storage iron and cellular iron needs, respectively. Combined with hematologic measurements, these 2 iron indexes are believed to provide a good picture of iron status (8). Indicators of iron deficiency are difficult to interpret in infants, however, because of the effect of coincident changes in physiology and metabolism during growth and development and because of frequent infections (9).

Cord SF has been shown to reflect neonatal iron stores (10). SF concentrations in the fetus increase throughout gestation (11), and at term, they are higher than those during most of postnatal life (12). However, the range of cord SF concentrations is quite wide (13), and normal reference limits are not available. The SF concentration changes markedly during the first year of life (14), although low cord SF has been associated with low SF later in infancy (15–17).

Studies of sTfR in the newborn are sparse, but cord sTfR is believed to reflect erythropoietic activity in the newborn (18). The value of cord sTfR in the assessment of iron status in the newborn has been questioned, however, because of the weak correlation to other iron indexes (19).

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Subjects

SUBJECTS AND METHODS

Subjects

A flow diagram of the study is given in Figure 1. This longitudinal study followed Norwegian children from birth until 2 y of age (20). Invitations were sent to 471 pregnant women of Norwegian or other Nordic descent who were registered to deliver at Aker University Hospital, Oslo, Norway, between April and June 1997. By the time of delivery, 78% of the invited women agreed to participate. Another 67 women who had not received the invitation were recruited at the maternity ward when they were admitted to the hospital for delivery. A total of 364 women fulfilled the following criteria: singleton birth, gestational period of 37–43 wk, and birth weight over the 2.5 percentile [2600 and 2700 g for girls and boys, respectively, according to Norwegian growth charts (21)]. The subject families were invited to participate again when the children were 6, 9, 12, 18, and 24 mo of age; blood sampling was done at ages 6, 12, and 24 mo. If a child had fever that suggested an ongoing infection shortly before planned blood sampling, a new appointment was made. A common cold (without fever) was not considered a sufficient reason to postpone the blood sampling. A total of 197 children (54% of those included at birth) participated on all 4 occasions of blood sampling.

After each visit, the families were provided with free diapers and other infant products but no food products. After each blood sampling, each mother was informed of her child’s iron status. Written informed consent was obtained from each child’s parent or parents. The study was approved by the Regional Committee for Research Ethics and the Norwegian Data Directorate.

Data collection

From the women’s pregnancy records, we collected the following data: infant’s gestational age and maternal smoking habits, body weight, height, SF concentration in early pregnancy, and lowest hemoglobin throughout pregnancy. Gestational age was based on estimated date of delivery by using an ultrasound scan carried out at the hospital 17–19 wk after the last menstruation. Smoking status was recorded as daily smoker, occasional smoker, or nonsmoker, and the number of cigarettes per day was registered. In the analyses, only 2 categories were used: daily smokers and nonsmokers (which included occasional smokers). Body mass index (BMI; in kg/m²) was calculated. In the current study, we used only first-trimester BMI values, which were based on weight measurements taken no later than week 14 of gestation.

Information about each mother’s background (ie, age, education, marital status, number of previous births, and number of years since last birth) and use of vitamin and mineral supplements during pregnancy was collected through interview and a questionnaire administered at the hospital 1–2 d after birth. The maternal intake of iron from supplements in each trimester was calculated with the use of a calculator on the basis of the collected information and product content. In the analyses, the average intake of iron from iron supplements during the second and third trimesters (or categories thereof) is reported, if not otherwise stated. Information on the child’s birth weight, length, head circumference, and sex was collected from the child’s medical record.

A questionnaire about sickness and fever in the child during the previous week or month was administered immediately before blood sampling at 6, 12, and 24 mo. Fever during the previous month was used as an indication of infection than may have an influence on SF values. Dietary assessment was performed at 6, 9, 12, 18, and 24 mo, but these data will be presented elsewhere.
Use of iron supplements in pregnancy and infancy

In Norway, there is no iron fortification of flour or other food items except baby foods. On the basis of SF measurements early in pregnancy, the pregnant women (n = 327) were given the following recommendations about iron prophylaxis (22, 23): 1) in the case of iron deficiency anemia (ie, hemoglobin < 110 g/L and SF < 20 μg/L), iron doses up to 100 mg Fe/d were recommended; 2) if SF concentrations were <20 μg/L, supplementation with 30–50 mg Fe²⁺/d was recommended; 3) if SF concentrations were 20–60 μg/L, supplementation with 30–50 mg Fe²⁺/d starting in week 20 was recommended; and 4) if SF concentrations were >60 μg/L, no extra iron was recommended.

Low iron status in the children at age 6 mo was defined as SF concentrations < 10 μg/L. At ages 12 and 24 mo, low iron status was defined as SF concentrations < 10 μg/L or as SF concentrations between 10 and 15 μg/L in combination with hemoglobin < 110 g/L (24). Low iron status was found in 2% (7/281) of infants at age 6 mo, 12% (29/249) of infants at age 12 mo, and 17% (38/229) of children at age 24 mo (20). It was recommended that these children be given a liquid iron preparation [(9 mg Fe/mL) Nycoplus Neo-Fer; Nycomed AS, Asker, Norway] at a dose of 2.5 mL twice a day —ie, 45 mg/d for 2 mo—to improve iron status. Women reporting use of iron supplements in pregnancy and children with low iron status are included in the statistical analyses, unless stated otherwise.

Blood sampling and blood analyses

At birth, cord blood samples (n = 364) were collected into serum separation tubes (Vacutainer SST; BD Diagnostic, Plymouth, United Kingdom) after the umbilical cord was clamped. The samples were placed in a refrigerator and centrifuged at 2500–3000 rpm for 10 min at room temperature (Model 203 centrifuge; Sigma Laborzentrifugen GmbH, Osterode, Germany), aliquoted, and frozen within 24 h. When the children were 6 (n = 287), 12 (n = 249) and 24 (n = 231) mo old, blood samples were drawn by hospital laboratory technicians and collected into Vacutainer SSTs and Vacutainer tubes containing EDTA (Vacutainer EDTA; BD Diagnostic). The blood samples were drawn from an antecubital vein after the use of an anesthetic (Vacutainer EDTA; BD Diagnostic). The blood samples from the Vacutainer SSTs and Vacutainer tubes containing EDTA (Vacutainer EDTA; BD Diagnostic). The blood samples were drawn from an antecubital vein after the use of an anesthetic cream (EMLA; AstraZeneca AS, Södertälje, Sweden). In a few cases (n = 12), a capillary sample was drawn from a fingertip when the child was 6 mo old. There was no significant difference in the mean iron indexes between the capillary and venous samples, and the capillary samples were thus included. Blood samples from the Vacutainer EDTA tubes were sent to the laboratory for measurements of the hematologic variables. The blood samples in the Vacutainer SSTs were kept at room temperature for ≤60 min and then centrifuged at 3000 rpm for 10 min at room temperature (Lavofuge 400; Heraeus GmbH, Osterode, Germany) and placed in a refrigerator. The serum fraction was collected and aliquoted within 24 h. The aliquots were stored at −70 °C until they were analyzed.

Analyses of iron indexes

Analyses of iron indexes were performed at Aker University Hospital; analyses of SF (180 Ferritin assay; Chiron Diagnostics ACS, Medfield, MA) were performed at the Central Laboratory, and those of sTfR (IDEA sTfR IEMA assay; Orion Diagnostica, Turku, Finland) were performed at the Hormone Laboratory. The number of measurements of SF and sTfR is lower than the total number of blood samples listed in Figure 1 because of limited sample volume in some cases. SF was measured in 363, 281, 249, and 229 children at 0, 6, 12, and 24 mo of age, respectively, and sTfR was measured in 350, 264, 242, and 226 children at those same ages. Measurements of SF and sTfR on all 4 occasions were available for 191 and 169 infants, respectively.

The between-day CV was <5% for the SF assay and 5–6% for the sTfR assay. When the children were 6, 12, and 24 mo old, red blood cell indexes including hemoglobin and mean corpuscular volume (MCV) were measured by using a Sysmex hematology instrument (model 8000/9000; Sysmex Corp, Kobe, Japan) with a between-day CV of <1%. Red blood cell indexes were not measured in cord blood because the hematology instrument was not suitable for the high viscosity of the blood. The central laboratory regularly underwent quality-control evaluations (Labquality Ltd, Helsinki, Finland). Internal quality controls were used for the sTfR analyses.

Statistical analysis

Basic maternal and child characteristics are reported as means ± SDs or proportions. Because of the skewed distribution of SF and sTfR, these results are presented as geometric means (and 95% CIs). The geometric mean is calculated by back-transformation of the mean of log-transformed data. For comparison with previously published data, we have also included mean (±SD) and median (25th–75th percentile, or quartile 1–quartile 3 (Q1–Q3). Values of SF and sTfR were log transformed in the statistical analyses when parametric methods were used. Calculation of the ratio of sTfR to log ferritin provided the sTfR-F index, as reported in tables. Maternal intake of iron supplements was not normally distributed, and a substantial number of the mothers had not taken iron supplements. Thus, for this variable, we report the median and Q1–Q3 values and used non-parametric tests, or we used categories of the average iron intake from supplements in the second and third trimesters (0, 1–30, and >30 mg/d).

Student’s t test and a chi-square test were used to compare independent groups. Bivariate correlations were examined by using the Spearman rank-order correlation test. Linear regression analyses and analyses of variance (ANOVA) were used to estimate the relative influence of various factors on cord SF and sTfR with control for potential confounders. Repeated-measures ANOVA was used for comparison of SF and sTfR in the different age groups. These statistical analyses were performed by using SPSS for WINDOWS software (version 12.01; SPSS Institute, Chicago, IL).

To assess the significance of the difference between 2 correlation coefficients, we employed Fisher’s r-to-z transformation by using the VassarStats Web Site for Statistical Computation (Internet: http://faculty.vassar.edu/lowry/VassarStats.html). To study possible interactions between the age and the sex of the child for SF, we used a linear model for repeated measurements (linear mixed models). The interaction between categories of cord SF (in quartiles) and the age or the sex of the child for the proportion of those with SF < 15 mg/L was analyzed by an extension of logistic regression to repeated measurements [generalized linear mixed models (25)]. The interaction analyses were performed with the free R software (version 2.4.0; Internet: http://www.r-project.org/) by using the lme and lmer functions in the nlme package (26). Gaussian generalized additive regression models, as implemented in S-PLUS for WINDOWS software.
TABLE 1
Characteristics of the population

<table>
<thead>
<tr>
<th></th>
<th>Subjects with available data</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>Mothers</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Age (y)                 | 364                          | 29.9 ± 4.4
| Living with partner [n (%)] | 364 | 338 (92.9) |
| Education ≥12 y [n (%)] | 364                          | 262 (72) |
| Parity                  |                              |       |
| Para 0 [n (%)]          | 364                          | 180 (49.5) |
| Para 1 [n (%)]          | 364                          | 150 (41.2) |
| Para 2+ [n (%)]         | 34                           | 34 (9.3) |
| Daily smoker [n (%)]    | 343                          | 62 (17) |
| First trimester BMI (kg/m²) | 250 | 23.5 ± 3.6 |
| Use of iron supplements |                              |       |
| Cord serum ferritin (SF) | 364 | 270 (74.2) |
| Second trimester intake (mg) | 363 | 9 (0–24) |
| Third trimester intake (mg) | 363 | 14 (0–33) |
| Early pregnancy ferritin (µg/L) | 327 | 52 ± 34 |
| Lowest pregnancy hemoglobin (mg/L) | 356 | 112 ± 11 |
| Newborns                |                              |       |
| Boys [n (%)]            | 364                          | 197 (54.1) |
| Gestational age (wk)    | 364                          | 40.1 ± 1.2 |
| Birth weight (g)        | 364                          | 3673 ± 455 |
| Birth length (cm)       | 351                          | 50.8 ± 2.0 |
| Head circumference (cm) | 360                          | 35.4 ± 1.3 |

1 For normally distributed variables, mean ± SD values are given.
2 Median; interquartile range (quartiles 1–3) in parentheses (all such values).

RESULTS

The characteristics of the mothers and newborns are listed in Table 1. Of the 364 mothers, 3 were 18–19 y old, and 5 were 40–42 y old. The mean birth weight was 3673 ± 455 g, and there was no significant difference between boys and girls. The mean birth length was 50.8 ± 2.0 cm, and there was a significant (P < 0.001) difference between the sexes: 51.2 ± 1.9 cm for boys and 50.4 ± 2.0 cm for girls.

Cord serum ferritin and serum soluble transferrin receptor in the total group and in boys and girls

Values of cord SF and cord stTfR are shown in Table 2, for the total group and for boys and girls separately. Geometric means (95% CIs) were 159 (148, 171) µg/L for SF and 7.3 (7.0, 7.6) mg/L for stTfR. The 5th–95th percentile reference intervals were 40–468 µg/L for cord SF and 3.8–14.9 mg/L for cord stTfR.

There was a significant difference in geometric mean cord stTfR between the sexes, with the boys having the higher values. Geometric mean cord SF did not differ significantly between the boys and the girls. However, significantly (P = 0.013) more boys than girls had cord SF values below the 5th percentile: 7.7% and 1.8%, respectively.

There was a negative correlation between cord concentrations of SF and stTfR (ρ = −0.21, P < 0.001), and low values of SF were thus associated with high values of stTfR. With SF values ≥100 µg/L, however, stTfR reached a plateau, as shown in Figure 2. Cord SF concentrations ≤100 µg/L were found in 19.0% of the samples; the proportion tended to be higher in boys than in girls (22.4% and 15.0%, respectively; P = 0.068).

Gestational age and anthropometric data at birth

Mean gestational age was 40.1 ± 1.2 wk, and there was no significant difference between the sexes. Gestational age was correlated with cord SF (ρ = 0.13, P = 0.016) and cord stTfR (ρ = 0.24, P < 0.001).

The values of cord SF, cord stTfR, and cord stTfR-F index (stTfR/log SF) and the weight and length of infants according to increasing lengths of gestation are shown in Table 3. There was a significant increase in cord stTfR from gestational age 37–38 wk through gestational age 42 wk. There was a parallel increase in cord SF until week 41, which was followed by a drop in week 42. This sequence of changes according to the length of gestation probably explains why there was a significant difference in cord SF by ANOVA, but not by linear trend analysis.

TABLE 2
Cord serum ferritin (SF), serum soluble transferrin receptor (stTfR), and the ratio of stTfR to SF (stTfR/log SF) in boys and girls, separately and combined

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>Boys</th>
<th>Girls</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF (µg/L)</td>
<td>n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>363</td>
<td>196</td>
<td>167</td>
<td></td>
</tr>
<tr>
<td>x ± SD</td>
<td>196 (127)</td>
<td>195 (135)</td>
<td>196 (117)</td>
<td>0.94</td>
</tr>
<tr>
<td>Geometric x (95 %CI)</td>
<td>159 (148, 171)</td>
<td>151 (137, 167)</td>
<td>168 (151, 187)</td>
<td>0.15</td>
</tr>
<tr>
<td>Median (Q1–Q3)</td>
<td>169 (114–242)</td>
<td>169 (104–245)</td>
<td>169 (117–240)</td>
<td>0.65</td>
</tr>
<tr>
<td>stTfR (mg/L)</td>
<td>n</td>
<td>350</td>
<td>187</td>
<td>163</td>
</tr>
<tr>
<td>x ± SD</td>
<td>8.0 (4.1)</td>
<td>8.7 (4.6)</td>
<td>7.3 (3.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>Geometric x (95 %CI)</td>
<td>7.3 (7.0, 7.6)</td>
<td>7.8 (7.4, 8.3)</td>
<td>6.7 (6.3, 7.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>Median (Q1–Q3)</td>
<td>7.1 (5.5, 9.6)</td>
<td>7.6 (5.8, 10.2)</td>
<td>6.5 (5.2, 8.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>stTfR/log SF</td>
<td>n</td>
<td>350</td>
<td>187</td>
<td>163</td>
</tr>
<tr>
<td>x ± SD</td>
<td>3.8 (2.6)</td>
<td>4.3 (3.1)</td>
<td>3.3 (1.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Geometric x (95 %CI)</td>
<td>3.4 (3.2, 3.5)</td>
<td>3.6 (3.4, 3.9)</td>
<td>3.1 (2.8, 3.3)</td>
<td>0.001</td>
</tr>
<tr>
<td>Median (Q1–Q3)</td>
<td>3.2 (2.4–4.4)</td>
<td>3.4 (2.6–4.8)</td>
<td>2.9 (2.3–4.0)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

† P for the difference between groups by Student’s t test (for mean or geometric mean) or Mann-Whitney U test (for median).

Interquartile range [quartile (Q) 1–3] in parentheses (all such values).
After adjustment for gestational age, there was no significant correlation between cord SF or cord sTfR and length, weight, or head circumference at birth.

Maternal factors

Iron indexes at birth were not correlated with the mother’s age, the number of her previous childbirths, or the number of years since her last delivery. No significant difference in iron indexes was found between mothers living with their partner and single mothers or between mothers with high (≥12 y) and low (<12 y) education. Maternal first-trimester BMI was correlated with cord sTfR (n = 240; r = 0.20, P = 0.001) but not to SF. In a linear regression analysis, a significant association between first-trimester BMI and cord sTfR remained significant (n = 229; partial r = 0.31, P < 0.001) after adjustment for birth weight, birth length, and gestational age.

Maternal smoking habits

Infant birth weight was 3614 ± 480 g in the group of smokers (n = 62) and 3692 ± 444 g in the group of nonsmokers (n = 281) (P = 0.22). Gestational age was 39.9 ± 1.3 versus 40.2 ± 1.1 wk, respectively, in those 2 groups (P = 0.16). The lack of difference in the values in infants born to smoking and nonsmoking mothers may be due to the exclusion of premature and low-birth-weight infants.

Infants born to mothers who reported daily smoking in early pregnancy (n = 61) had lower cord SF than did infants born to nonsmoking mothers (n = 281), even after adjustment for gestational age and birth weight [geometric mean: 134 µg/L (95% CI: 112, 159 µg/L) and 166 µg/L (95% CI: 153, 180 µg/L), respectively (P = 0.025)]. Further analyses showed that there was no dose effect of smoking on cord SF in smokers. There was no difference in sTfR according to smoking status.

There was no significant difference in early pregnancy SF between smoking and nonsmoking women, but women who smoked during pregnancy had a slightly lower median and Q1–Q3 intake of iron from supplements [9.0 (0–22.8) mg/d] than did nonsmokers [14.0 (2.1–29.9) mg/d] (P = 0.033). In a regression model that included smoking, gestational age, birth weight, and categories of intake of iron from supplements, smoking had a significant, independent negative association with cord SF. The effect remained significant after adjustment for the use of cod liver oil as indicator of healthy lifestyle (27): the geometric mean cord SF was 135 (95% CI: 113, 161) µg/L and 166 (95% CI: 153, 180) µg/L in smokers and nonsmokers, respectively (P = 0.035).

Iron status in pregnancy

A total of 38 of 327 mothers (11.6%) with SF measurement had low iron stores, as indicated by SF < 20 µg/L. Of these 38, 17 also had a low hemoglobin (<110 g/L) at one point during pregnancy. Probably because of the selective iron prophylaxis, maternal SF concentrations in early pregnancy were not correlated with cord SF or with cord sTfR. Furthermore, there was no difference in cord SF or cord sTfR between infants born to mothers with low (SF < 20 µg/L) or adequate (>60 µg/L) iron stores in early pregnancy or between infants born to mothers with anemia (defined as hemoglobin < 110 g/L; n = 139) or without anemia [(1) n = 217] at ≥1 pregnancy check-ups. The lowest hemoglobin measurement in pregnancy was, however, weakly correlated to cord SF (r = 0.11, P = 0.04).

Intake of iron supplements in pregnancy

Infants born to mothers who had taken iron supplements during pregnancy (n = 270) had significantly (P = 0.02) higher cord SF concentrations than did infants born to mothers who had not taken iron supplements (n = 93). The geometric mean (and 95% CI)

<table>
<thead>
<tr>
<th>TABLE 3 Cord serum ferritin (SF), serum soluble transferrin receptor (sTfR), ratio of sTfR to SF (sTfR/log SF), birth weight, and birth length in relation to gestational age</th>
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<tr>
<td>SF (µg/L)</td>
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<td>32</td>
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<td>sTfR (mg/L)</td>
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<td>31</td>
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<tr>
<td>sTfR/log SF</td>
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<td>Weight (g)</td>
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<td>32</td>
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<tr>
<td>Length (cm)</td>
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<td>31</td>
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</table>

1 Geometric mean; 95% CI in parentheses (all such values).
CI) values were 168 (155, 183) µg/L and 134 (116, 154) µg/L, respectively.

In accordance with the recommendations for selective iron prophylaxis, the intake of iron from supplements differed according to maternal SF. In women with SF concentrations < 20 µg/L (n = 38), the median and Q1–Q3 intake of iron from supplements was 33 (17–82) mg/d, whereas it was 14 (3–27) mg/d in women with SF concentrations of 20–60 µg/L (n = 192). In the group with adequate iron stores (SF > 60 µg/L; n = 96), it was only 1 (0–14) mg/d. There were significant differences among the 3 groups both by Kruskal-Wallis test and by pairwise comparisons with the Mann-Whitney U test (P < 0.001 for all). Only 2 mothers with SF < 20 µg/L did not take iron supplements.

The intake of iron supplements during pregnancy did not predict cord sTfR. Cord SF, however, was positively correlated with iron intake from supplements (ρ = 0.18, P < 0.001), and there was a significant difference in cord SF according to 3 categories of iron intake (P = 0.009). In the group not taking iron supplements (n = 97), geometric mean (95% CI) cord SF was 133 (116, 152) µg/L. In the group taking 1–30 mg Fe/d (n = 182), cord SF was 166 (150, 183) µg/L, and, in the group taking >30 mg Fe/d (n = 83), it was 179 (154, 207) µg/L. Thus, infants born to women taking iron supplements had significantly (P = 0.003) higher cord SF concentrations than did infants born to women not taking supplements, despite the lower maternal SF. In contrast, there was no significant difference in cord SF between infants born to mothers with a high or a low intake of iron from supplements.

Predictors of serum ferritin and serum soluble transferrin receptor concentrations in multivariate analyses

In a stepwise linear regression (n = 337), we included the following factors that we found to influence cord SF: gestational age, sex of the child, smoking status (daily smokers versus nonsmokers), iron intake from supplements (3 categories), and lowest value of hemoglobin in pregnancy. For cord SF, a significant age interaction was obtained for smoking (partial r = –0.12, P = 0.031), and a positive association was found for iron intake from supplements (partial r = 0.16, P = 0.004). When cord sTfR was added to the model (n = 324), significant negative associations were found for smoking during pregnancy (partial r = –0.12, P = 0.028) and cord sTfR (partial r = –0.32, P < 0.001), and significant positive associations were obtained for gestational age (partial r = 0.16, P = 0.005) and iron intake from supplements (partial r = 0.13, P = 0.016).

In a stepwise linear regression analysis for sTfR, we included gestational age, sex, and first-trimester BMI (n = 241) in the model. Positive associations were obtained for gestational age (partial r = 0.24, P < 0.001), male sex of the child (partial r = 0.19, P = 0.004), and first-trimester BMI (partial r = 0.30, P < 0.001). The inclusion of cord SF in the model did not change these associations, but there was an independent negative association with cord SF (partial r = –0.26, P < 0.001).

Influence of iron status at birth on iron status at age 6, 12, and 24 mo

In both sexes, there was a significant (P < 0.001, repeated-measures ANOVA) decline in SF from birth to age 6 mo and a further but more modest decline to ages 12 and 24 mo (Figure 3). Likewise, sTfR concentrations declined significantly (P < 0.001) from birth to age 6 mo in both sexes and changed modestly thereafter. At 6 mo, but not at 12 and 24 mo, boys had significantly lower geometric mean SF and significantly higher sTfR concentrations (P < 0.001 for both) than did girls.

Cord sTfR did not correlate with SF or sTfR at age 6, 12, or 24 mo. Neither cord SF nor cord sTfR was correlated with hemoglobin or MCV at any age. A comparison of infants with cord SF < 100 µg/L and those with cord SF > 100 µg/L showed no difference in hemoglobin, MCV, or sTfR at age 6, 12, or 24 mo. In contrast, there was a strong positive correlation between cord SF and SF at ages 6, 12, and 24 mo (Table 4). The exclusion of children with fever during the month before blood sampling (25.2%, 41.3%, and 42.0% at ages 6, 12, and 24 mo, respectively) or of children with low iron status at 6 or 12 mo (2% and 12%, respectively) did not change the overall pattern. Hence, all children were included in the analyses.

Age 6 mo

The correlation between cord SF and SF at age 6 mo was significant in both sexes (ρ = 0.37 for girls and 0.55 for boys;

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Figure 3. Geometric mean (95% CI) serum ferritin and serum soluble transferrin receptor from birth to age 24 mo in girls (○) and boys (●). Analyses used a linear model for repeated measurements. For serum ferritin (n = 191), there were significant results for the age (P < 0.001) and the sex (P = 0.002) of the child but no significant age × sex interaction (P = 0.33). For serum soluble transferrin receptor (n = 169), there were significant results for the age (P < 0.001) and the sex (P = 0.002) of the child but no significant age × sex interaction.
P < 0.001). At 6 mo, 17 of 281 infants (6%) had SF concentrations < 15 µg/L. Sixteen of 17 infants with SF < 15 µg/L were boys. Infants with cord SF concentrations < 100 µg/L were significantly (P < 0.05) more likely to have SF concentrations < 15 µg/L at 6 mo (7/50; 14.0%) than were infants with cord SF concentrations ≥ 100 µg/L (10/230; 4.3%), and the geometric mean SF in the former group also was significantly (P < 0.001) lower—29 µg/L (24, 35 µg/L) compared with 50 µg/L (46, 55 µg/L). The relation between cord SF and SF at age 12 mo was investigated further by comparing the quartiles of cord SF values with the proportion with SF < 15 µg/L at age 6 mo (Figure 4). Children born with cord SF in the lowest quartile had a significantly greater risk of low SF at age 6 mo (P < 0.001) than did those with cord SF in the highest quartile, a pattern that was most apparent in boys. Adjustment for cord sTfR did not change the finding.

**Age 12 mo**

The correlation between cord SF and SF at age 12 mo was significant in both sexes (ρ = 0.35 for girls and 0.30 for boys; P < 0.001). At age 12 mo, 53 of 249 infants (21.3%) had SF concentrations < 15 µg/L. There was no difference by sex in the proportion of infants with SF concentrations < 15 µg/L (24, 35 µg/L) compared with 50 µg/L (46, 55 µg/L). The mean cord SF concentrations observed in the present study agree with results from other studies (15, 28, 29). In the present cohort, 11% of subjects had cord SF concentrations < 76 µg/L—values that were associated in one study with lower mental and psychomotor test scores at age 5 y (30). Given that low iron status early in life may affect mental, neurologic, and motor functions later in childhood (1), the proportion of such subjects in the present study gives reason for concern, even though a causal relation between cord iron status and later neurodevelopmental outcome has not been established (13).

The cord sTfR concentrations observed in the present study were higher than those found in other surveys (18, 31, 32). This difference may be due to differences in methods, or it may reflect true differences in the population. As was reported by others (19, 33), we too found an inverse correlation between cord SF and cord sTfR. Our data reveal a sharp increase in sTfR when cord SF declines below 100 µg/L, which suggests that that value could be an appropriate cutoff for cord SF. In our healthy, term children, nearly 20% had cord SF concentrations below this threshold. Further studies are required to establish the association between iron status at birth and later health and development.

### Table 4

<table>
<thead>
<tr>
<th></th>
<th>Cord SF</th>
<th>Cord sTfR</th>
<th>Cord sTfR/log SF</th>
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<td></td>
<td>n</td>
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<tr>
<td>SF</td>
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<tr>
<td>6 mo</td>
<td>280</td>
<td>0.45</td>
<td>&lt; 0.001</td>
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<tr>
<td>12 mo</td>
<td>248</td>
<td>0.31</td>
<td>&lt; 0.001</td>
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<tr>
<td>24 mo</td>
<td>228</td>
<td>0.16</td>
<td>0.017</td>
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<tr>
<td>6 vs 12 mo</td>
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<td>0.062</td>
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<td>6 vs 24 mo</td>
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<td></td>
<td>&lt; 0.001</td>
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<td>12 vs 24 mo</td>
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<td>0.085</td>
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<tr>
<td>sTfR</td>
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<tr>
<td>6 mo</td>
<td>263</td>
<td>−0.16</td>
<td>0.011</td>
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<tr>
<td>12 mo</td>
<td>241</td>
<td>0.01</td>
<td>0.87</td>
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<tr>
<td>24 mo</td>
<td>225</td>
<td>0.01</td>
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<td>6 vs 12 mo</td>
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<td>6 vs 24 mo</td>
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<td>12 vs 24 mo</td>
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* sTfR/log SF is also called the sTfR-F index.
showed the following significant associations: age of the child (age group is shown in Figure 3. Analysis by generalized linear mixed models cord serum ferritin in the total group. The number of boys and girls in each
ferrin receptor expression on all cell types, which is reflected in
fetal life, rapid cell proliferation and tissue growth affect trans-
membranes during periods of high erythropoietic activity (34). In
presence of a greater amount of transferrin receptors on cell
(19, 33). An increase in the sTfR concentrations reflects the
pregnancy. A positive association between sTfR and gestational
Length of gestation

We found an increase in sTfR throughout the last weeks of
pregnancy. A positive association between sTfR and gestational
length has been reported in some studies (32) but not in others
(19, 33). An increase in the sTfR concentrations reflects the
presence of a greater amount of transferrin receptors on cell
membranes during periods of high erythropoietic activity (34). In
fetal life, rapid cell proliferation and tissue growth affect trans-
ferrin receptor expression on all cell types, which is reflected in
the cord sTfR concentration and which may explain the increase
with greater gestational age (19).

As observed in other studies (11, 35, 36), cord SF concentra-
tions increased with gestational age, except for weeks 42 and 43.
The placenta plays an important role in the regulation of the
accumulation of fetal iron stores (35). Toward the end of the third
trimester, the placenta accumulates ferritin, which may advance
placental iron delivery to the fetus (37). The decline in cord SF
after gestation week 41 may be caused by degeneration of the
placenta (38).

Sex and iron status at birth and early childhood

We found that boys had significantly higher cord sTfR con-
centrations than did girls, which may reflect greater erythropoi-
etic activity in boys than in girls (18). During infancy, higher
sTfR in boys than in girls has been found in some (31, 39) but not
all (40) studies. Because the difference in sTfR is present at birth,
we speculate that it could be due to hormonal factors: in male
embryos, the testosterone concentration is high from early in
gestation (41). The fact that the administration of sex hormones
to adults changes sTfR values (42) indicates a hormonal effect on
this variable.

In our study, cord SF concentrations in boys did not differ
significantly from those in girls. This finding agrees with results
from some (15, 18) but not all (43) studies. We observed, how-
ever, that low cord SF (<40 µg/L) was more prevalent in boys
than girls, which suggests that boys are already at risk of low iron
status at birth.

We found that cord SF was associated with SF at ages 6, 12,
and 24 mo. In smaller studies, low SF in early infancy has been
shown to persist later in infancy (15–17). Our data show that the
risk of low SF at age 6 mo was most apparent in boys—in
particular, boys born with cord SF concentrations below the
median. At age 12 mo, the sex difference was no longer apparent,
but in both sexes, the risk of low iron stores was influenced by
cord SF. Thus, low iron stores at birth place children at greater
risk of compromised iron status in the first years of life. In the
present study overall, the cord sTfR concentration did not have
the same predictive value as did the cord SF concentration in iden-
tifying those persons at risk of low iron stores later in infancy.

Previous reports showed that, in early childhood, girls have
iron index values that are consistent with better iron status than
those in boys (39, 44–46). That finding was also reported pre-
viously from the present cohort (20): at age 6 mo, the girls had
significantly higher values of hemoglobin, MCV, and SF than
did the boys. At ages 12 and 24 mo, the difference in MCV
remained significant.

Maternal factors

First-trimester BMI was positively associated with cord sTfR.
Even if not particularly strong, the association remained the most
important maternal predictor of cord sTfR, even after adjustment
for potential confounders. We have no explanation for this find-
ing, but we can speculate that it is due to hormonal or lifestyle
factors. This finding needs to be confirmed.

In line with previous studies (47, 48), we found that infants
born to smoking mothers had significantly lower cord SF con-
centrations than did infants born to nonsmoking mothers. The
various possible explanations include hypoxia leading to in-
creased erythrocyte production (49, 50), impaired uterine blood

![FIGURE 4. Proportions of boys (□) and girls (□) with cord serum ferritin concentrations <15 µg/L at ages 6, 12, and 24 mo according to quartiles of cord serum ferritin in the total group. The number of boys and girls in each age group is shown in Figure 3. Analysis by generalized linear mixed models showed the following significant associations: age of the child (P < 0.001), sex (P = 0.010), and children with cord serum ferritin in quartiles 1 (P < 0.001), 2 (P < 0.001), and 3 (P = 0.002) relative to quartile 4. Furthermore, there was a significant interaction between the age of the child and quartile 1 (P = 0.006) and quartile 2 (P = 0.043) cord serum ferritin concentrations.]
flow or interference with transplacental availability of iron (51, 52), shortening of gestational age, reduction in birth weight, and less healthy eating habits or less use of iron supplements (53, 54). In the present study, we found a modestly lower intake of iron from supplements in smoking women. The smoking effect did, however, remain significant after adjustment for potential confounders, which suggests an independent effect of smoking on cord SF.

Maternal iron status and iron supplement use

We did not find a significant association between maternal SF concentrations and cord SF or cord sTfR concentrations. This is not surprising, given that the women were advised to use iron supplementation according to their SF values. Thus, in the present study, low iron stores during pregnancy probably were compensated for by the use of iron supplements in the last 2 trimesters. The use of iron supplements during pregnancy has been debated. Iron supplementation improves maternal iron status and pregnancy outcome when the mother has low iron status; however, prophylactic supplementation of iron-replete women may increase the risk of complications and oxidative stress (55). Some reports suggest that it is only when the mother is severely iron deficient that her iron status or use of supplements affects her infant (9, 12). In the present population, however, with a fairly good overall iron status, maternal use of iron supplements was an independent predictor of cord SF concentrations, and the children of mothers not taking iron supplements had the lowest cord SF concentrations. The fact that the children with modestly low SF concentrations at birth remained at high risk of low SF concentrations during the first year of life suggests that the use of iron supplements by pregnant women with normal iron stores also should be considered.

Strengths and limitations of the study

The strengths of the present study include the size of the population, the data available from pregnancy, the measurements of both SF and sTfR, and the fact that most of the children were followed until age 2 y. Our study was confined to healthy term infants and excluded premature and low-birth-weight infants—ie, infants at greater risk of low iron status at birth (24). Thus, our findings cannot be generalized to these high-risk infants. Furthermore, this observational study can only show associations, which are no proof of causality. Some of the associations are relatively weak, but because they are related to lifestyle and they can potentially be modified, they may be important.

The use of iron supplements was recommended to pregnant women with low SF concentrations. Thus, we cannot properly evaluate the value of iron supplements during pregnancy. Information on maternal iron supplement use was collected 1–2 d after delivery. Retrospectively collected data are uncertain, but, if anything, poor data quality tends to weaken the associations.

The inclusion of measurements from children for whom iron supplements at age 6 or 12 mo were recommended to improve iron status, as well as from all children regardless of recent infections, may have weakened the associations. However, the exclusion of children with low iron status at age 6 or 12 mo or children with fever during the previous month at age 6, 12, or 24 mo did not alter the patterns.

Finally, statistical models showed that there was no significant interaction between iron status and sex, but that boys in general had poorer iron status than girls. The descriptive data, however, suggest that the sex differences in SF and sTfR were most apparent during the first 6 mo. Ascertainment of whether boys have lower iron status than girls from birth to age 2 y or only during a period of infancy will require larger studies.

In conclusion, these data suggest that the cord SF concentration is a strong predictor of iron status during the first 2 y of life, and that cessation of smoking and adequate iron prophylaxis during pregnancy may improve iron status at birth. Compared with girls, boys are at greater risk of low iron status at birth and in early infancy. Given the potentially serious consequences of either low or excess iron intake during pregnancy and infancy, our data suggest that selective low-dose iron prophylaxis during pregnancy, after measurement of the SF concentration, may be a valuable approach to optimizing the iron status of both mother and child.

We are indebted to the families who participated in the study, the laboratory technicians at Aker University Hospital under the leadership of Brit Eieland, and several project workers and graduate students who contributed considerably to the study. We especially thank Kerstin Trygg, Marianne Hope, Gunn Helene Arsky, Janne Liabo, Nina Cecilie Øverby, Torild Lilieaas Gronnerud, Ellen Margrethe Hovland, Kirsti Kverndokk, and Kathrine C. Sorensen for their invaluable contribution to the data collection; Abderrahim Oulhaj for statistical help and advice on the interaction analyses; and Eha Nurk for assistance in the preparation of the manuscript. We greatly appreciate Elfrid Blomdal’s help with the literature search.

The authors’ responsibilities were as follows—GH: design of the experiment, collection and analysis of data, and writing of the manuscript; HR: analysis of data and writing of the manuscript; AW: design of the experiment, analysis and interpretation of data, and critical revision of the manuscript; ELM: collection and analysis of data; EH: analysis of data; BB-I: design of the experiment, analysis and interpretation of the data, and critical revision of the manuscript; and all authors: reading and approval of the final manuscript. None of the authors had a personal or financial conflict of interest.

REFERENCES

IRON STATUS IN INFANCY


Comparison between plasma and erythrocyte fatty acid content as biomarkers of fatty acid intake in US women

Qi Sun, Jing Ma, Hannia Campos, Susan E Hankinson, and Frank B Hu

ABSTRACT

Background: Erythrocyte fatty acids may be superior to plasma fatty acids for reflecting long-term fatty acid intake because of less sensitivity to recent intake and a slower turnover rate.

Objective: The objective was to compare the fatty acid content of erythrocytes with that of plasma with respect to their abilities to reflect usual fatty acid intake.

Design: Fatty acids in plasma and erythrocytes were measured by capillary gas-liquid chromatography in 306 US women aged 43–69 y. Fatty acid intake was assessed with a food-frequency questionnaire, which was validated for measuring intakes of various fatty acids.

Results: Docosahexaenoic acid (DHA, 22:6n–3) in erythrocytes and plasma provided the strongest correlations with its intake, but erythrocyte DHA concentrations (Spearman’s partial correlation coefficient \( r_s = 0.56 \)) were better than plasma DHA concentrations \( (r_s = 0.48) \) as a biomarker. Total trans fatty acids \( (r_s = 0.43) \) and total 18:1 trans isomers \( (r_s = 0.42) \) in erythrocytes were also more strongly correlated with intake than were those in plasma \( (r_s = 0.30 \) and \( r_s = 0.29 \), respectively). Moderate correlations were observed for linoleic acid \( (18:2n–6; \text{erythrocytes}, r_s = 0.24; \text{plasma, } r_s = 0.25) \), α-linolenic acid \( (18:3n–3; \text{erythrocytes, } r_s = 0.18; \text{plasma, } r_s = 0.23) \), and eicosapentaenoic acid \( (20:5n–3; \text{erythrocytes, } r_s = 0.38; \text{plasma, } r_s = 0.21) \). For polyunsaturated and trans fatty acids, correlations between intakes and biomarkers improved moderately when average intakes over previous years were used.


KEY WORDS Fatty acids, erythrocytes, plasma, biological markers, food-frequency questionnaires, US women

INTRODUCTION

Precise assessment of fatty acid intake is essential for nutritional epidemiologic studies. In contrast with self-report methods, eg, food-frequency questionnaires (FFQs) and diet records, biomarkers of dietary fatty acids have unique strengths. Biomarkers are objective and do not rely on the accuracy of memories, awareness of fat intake, or willingness to report details of diet (1, 2). Also, nutrient databases may not adequately reflect temporal changes in food composition, which could be readily accommodated by biomarkers (2). Therefore, measurement errors of biomarkers are largely independent of those of self-report methods. For these reasons, biomarkers of fatty acid intake have been widely used in epidemiologic studies to validate FFQs (3–8), to evaluate compliance with dietary interventions (9, 10), or to predict risk of diseases (11–14).

Fatty acids that are of largely exogenous origin, ie, n–3, n–6, trans, and odd-numbered fatty acids, could provide the best quantitative estimate of their intakes (15). These fatty acids can be measured in various blood fractions and tissues, eg, plasma or serum, erythrocytes, and adipose tissue (1, 15). The half-life of linoleic acid in adipose tissue has been estimated to be 680 d (16), which indicates that fatty acids in this tissue could be used to reflect long-term fat intake. Studies have corroborated that polyunsaturated fatty acids (PUFAs) and trans fatty acids in adipose tissue were reasonably correlated with intake measured by FFQs (4, 5, 17, 18). However, the availability of adipose tissue limits its use in epidemiologic studies (15). Instead, blood specimens are more available and widely used. One well-conducted controlled dietary trial clearly showed that serum n–3 fatty acid concentrations responded more quickly than did erythrocyte n–3 fatty acid concentrations to recent dietary supplementation with fish oil (19). Whether erythrocytes better reflect long-term dietary fatty acid intakes than does plasma or serum is of interest. Several studies have examined plasma or erythrocytes separately, and a range of correlations (0.20–0.80) was observed between concentrations of PUFAs and trans fatty acids in these specimens and intakes measured by various FFQs (3, 20–25). However, few studies have been conducted to compare correlations with plasma and erythrocytes within the same population. True between-person variation in diet, laboratory measurement errors, different dietary measurement methods used, and biological variability all contributed to the correlations observed in different studies, which makes it hard to draw conclusions about the

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2 Supported by research grants CA49449, CA42182, HL24074, HL34594, and CA87969 from the National Institutes of Health. FBH is a recipient of the American Heart Association Established Investigator Award.

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relative performance of these 2 specimens on the basis of current literature.

To overcome these limitations, in a US population of women we measured >30 fatty acids in both plasma and erythrocytes under identical conditions and compared them with respect to their ability to reflect long-term fatty acid intake assessed by validated FFQs.

SUBJECTS AND METHODS

Study population

The Nurses’ Health Study, initiated in 1976, consisted of 121,700 female registered nurses aged 30–55 y living in 1 of 11 US states. Data on the occurrence of cardiovascular diseases and other illnesses and major risk factors have been collected with biennial questionnaires since baseline. Between 1989 and 1990, blood samples were collected from 32,826 women. Within this subcohort, 167 cases of coronary heart disease newly diagnosed between 1990 and 1996 were identified, and 334 control subjects matched for age, smoking, fasting status, and time of blood drawing were selected. All cases and controls were free of major cancers and cardiovascular diseases at the time of blood drawing. This analysis was conducted in the control group. Of the 334 participants, 7 had missing plasma or erythrocyte measurements, 14 had missing information on intake of fat, and 7 were currently using fish-oil supplements. After these participants were excluded, 306 were available for analysis.

All participants gave written informed consent. The study protocol was approved by the Institutional Review Board of the Brigham and Women’s Hospital and the Human Subjects Committee Review Board of Harvard School of Public Health.

Dietary assessment

Fatty acid intake assessed with a semiquantitative FFQ in 1990 was primarily used as usual intake in this study; a detailed description of the FFQ was published elsewhere (1). In the Nurses’ Health Study, diet has been assessed with the FFQs since 1980 and updated every 4 y. The original 61-item FFQ used in 1980 was expanded to include >130 food items in the 1984, 1986, and 1990 FFQs. These FFQs inquired about food consumption in the previous year and about the use of cooking oil and fat for frying and baking and the addition of margarine and butter to food. For each food item, a standard portion size was specified, and the participants were asked how often, on average, they consumed foods of that specified amount during the previous year. There were 9 possible coding responses, ranging from “never or less than once per month” to “6 or more times per day.” Fatty acid intake was calculated by multiplying the frequency of consumption of each food by the fatty acid composition in the specified amount of that food. The contributions across all foods were then summed for each fatty acid. The food-composition database was primarily based on US Department of Agriculture (USDA) publications (26). Food-composition data from other publications and individual laboratories were also used to supplement the USDA data (27–29). The intake of major saturated fatty acids (12:0, 14:0, 16:0, and 18:0), monounsaturated fatty acids (MUFAs; 16:1n–7, 18:1n–9, and 20:1n–12), polyunsaturated fatty acids (PUFAs; 18:2n–6, 20:4n–6, and 18:3n–3), and trans fatty acids (16:1, 18:1, and 18:2 trans isomers) was calculated. In our FFQs, 3 questions inquired about intake of canned tuna fish, dark meat fish, and other fish. The assessment of intake of n–3 fatty acids of marine origin, ie, eicosapentaenoic acid (EPA, 20:5n–3), docosapentaenoic acid (22:5n–3), and docosahexaenoic acid (DHA, 22:6n–3), was based on these questions.

Both the 61-item FFQ and the expanded FFQs used in the Nurses’ Health Study were validated against multiple-week diet records and biomarkers in adipose tissue (1, 4, 17, 30). For example, among 92 Nurses’ Health Study participants, correlation coefficients between intakes assessed by the 1986 FFQ and multiple diet records were 0.57 for total fat, 0.68 for total saturated fatty acids, 0.48 for total PUFAs, and 0.58 for total MUFAs (1).

Blood sample collection and analysis

In 1989 and 1990, interested women in the Nurses’ Health Study were sent supplies needed to collect blood samples. The samples were sent back on ice by a prepaid overnight courier. Ninety-seven percent of the samples were received within 24 h of blood drawing. Immediately on arrival, the samples were centrifuged (1200 × g for 15 min at room temperature) and then divided into aliquots of plasma, erythrocytes, and Buffy coat fractions. These aliquots were stored in liquid nitrogen freezers at −130 °C or colder until analysis in 2000 and 2002. The methods used to collect and store blood samples have been proven reliable (31). Fatty acids in serum phospholipids stored at −80 °C for 7–12 y showed minimal degradation over time (32).

Our blood samples were stored at a much lower temperature (−130 °C), which was intended to minimize any influences on fatty acid concentrations caused by the long-term storage.

Fatty acid concentrations were determined by gas-liquid chromatography. The methods were described elsewhere (20). Briefly, fatty acids in plasma and erythrocytes were first extracted into isopropanol and hexane and then transmethylated with methanol and sulfuric acid. Fatty acid methyl esters were evaporated and redissolved in isooctane and then measured by gas-liquid chromatography. Individual peaks were identified by comparison with known standards, and each peak was quantified by calculating the area under the peak. The concentration of each individual fatty acid was expressed as a percentage of total area under the peaks.

Of 51 fatty acids identified, the 37 fatty acids in both plasma and erythrocytes that had meaningful concentrations (mean concentration >0.01%) are reported here. For the current analysis, MUFAs and PUFAs include cis isomers only. Trans isomers were reported separately. Within-run CV percentages were assessed by repeatedly analyzing pooled samples. The CV percentages of the most abundant fatty acids in plasma were generally lower than those in erythrocytes, although they were all reasonably low. For example, the CV percentage for palmitic acid (16:0) was 1.4% (plasma) compared with 3.3% (erythrocytes); for oleic acid (18:1n–9), 2.4% (plasma) compared with 2.8% (erythrocytes); for linoleic acid (18:2n–6), 1.8% (plasma) compared with 2.8% (erythrocytes); and for DHA, 3.4% (plasma) compared with 7.2% (erythrocytes). The CV percentages of trans fatty acids, for which the concentrations were relatively low, were higher than those of the more abundant fatty acids. The average CV percentage of 18:1 trans isomers was 8.0% for plasma and 7.6% for erythrocytes and of 18:2 trans isomers was 6.9% for plasma and 10.0% for erythrocytes.
Statistical analyses

Fatty acid intake measured with the 1990 FFQ was expressed as a percentage of total fat intake to be comparable with the biomarker measurements. For FFQ measurements and biomarkers, total intakes of saturated fatty acids, MUFAs, PUFAs, and trans fatty acids were calculated by summing the concentrations of individual fatty acids of the same class, if they were detectable and available. Crude and partial Spearman’s rank-correlation coefficients ($r_s$), adjusted for age at blood collection, fasting status (yes or no), BMI (in kg/m$^2$), current weight (in kg), postmenopausal status (yes or no), postmenopausal hormone use (never, past, and current), smoking status (never smoker, past smoker, current smoker of 1–14 cigarettes/d, current smoker of 15–24 cigarettes/d, and current smoker of ≥25 cigarettes/d), and the periods during which the blood samples were assayed, were calculated to determine correlations between fatty acid composition in plasma or erythrocytes and intake. We used $t$ tests to examine the significance of Spearman’s partial correlation coefficients (33). Correlation coefficients were considered significant at the 0.05 level. To compare the Spearman’s partial correlation coefficients between plasma and erythrocytes, we first obtained the residuals of biomarkers and dietary intakes corrected for the covariates. The residuals were ranked and then converted to probit scale to normalize the ranks (34). On the basis of these transformed ranks, Wolfe’s test for comparing dependent correlation coefficients was applied to test the hypothesis that correlation coefficients with dietary intakes were equal for plasma fatty acids and erythrocyte fatty acids (35).

Intake of fatty acids was also measured in 1984 and 1986 with the use of FFQs that were similar to the 1990 FFQ. Spearman’s partial rank-correlation coefficients between intake assessed in these years and the biomarker concentrations were calculated. We also calculated average fatty acid intakes by using the 1984, 1986, and 1990 FFQ measures. Correlations among fatty acids within plasma and erythrocyte measurements and correlations between plasma and erythrocyte fatty acids were also calculated.

Multivariate linear regression was used to detect linear trends of biomarker concentrations across deciles of average intake calculated from all 3 FFQs. Fatty acid concentration was entered into the model as a dependent variable; deciles of average intake and total energy intake, age, smoking status, BMI, current weight, postmenopausal hormone use, fasting status, and period of blood assay were entered as independent variables. Least-squares means of biomarkers were calculated for each decile of intake. Robust estimators of variance for these means were calculated to allow for the deviation from assumption of normal distribution of dependent variables (36). $P$ values for linear trend were calculated by entering the median values of each decile of intake into the models as a continuous variable. All $P$ values were 2-sided. Data were analyzed with the Statistical Analysis System software package (version 9.1; SAS Institute, Cary, NC).

RESULTS

Baseline demographic characteristics and intakes of the study participants are shown in Table 1. As expected, the women included in this study were older and more likely to smoke than were the overall Nurses’ Health Study population because they were selected as controls matched for the age and smoking status of the cases with myocardial infarction. Intakes of fatty acids and plasma and erythrocyte fatty acid concentrations are shown in Table 2.

Spearman’s partial correlation coefficients of plasma or erythrocyte fatty acids with intake in 1990 adjusted for age, fasting status, BMI, postmenopausal status, smoking status, and other covariates are shown in Table 3. We did not observe substantial differences between crude and adjusted correlation coefficients. For both plasma and erythrocytes, correlation coefficients with dietary saturated fatty acids and MUFAs were weak ($r_s \leq 0.20$). Of the PUFAs, DHA had the strongest correlations, albeit the correlation was stronger for erythrocytes ($r_s = 0.56$) than for plasma ($r_s = 0.48$). Similarly, EPA in erythrocytes was more strongly correlated with intake ($r_s = 0.38$) than was EPA in plasma ($r_s = 0.21$). Linoleic acid provided the third strongest correlations with intake assessed in 1990 adjusted for age, fasting status, BMI, current weight, postmenopausal hormone use, and erythrocytes barely differed ($r_s = 0.25$ and $r_s = 0.24$, respectively). The correlation coefficients for arachidonic acid (20:4n–6) and docosapentaenoic acid were close to zero. Total trans fatty acids ($r_s = 0.43$) and total 18:1 trans isomers ($r_s = 0.42$) in erythrocytes were more strongly correlated with intake than were those in plasma ($r_s = 0.30$ and $r_s = 0.29$, respectively). The correlations for the other trans isomers were not different between erythrocytes and plasma.

To examine the time integration of the biomarkers, we further calculated Spearman’s partial correlation coefficients between intake measured in 1984, 1986, and 1990 and the cumulative

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Baseline characteristics of study participants in the Nurses’ Health Study, 1990$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristics</td>
<td>Study participants ($n = 306$)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>60 ± 6$^4$</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>25.4 ± 4.5</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164 ± 6</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68 ± 13</td>
</tr>
<tr>
<td>Total energy (MJ/d)</td>
<td>7.5 ± 2.3</td>
</tr>
<tr>
<td>Carbohydrates (% of energy)</td>
<td>50.2 ± 8.7</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>18.6 ± 3.1</td>
</tr>
<tr>
<td>Total fat (g/d)</td>
<td>63.6 ± 25.2</td>
</tr>
<tr>
<td>(% of energy)</td>
<td>31.7 ± 6.4</td>
</tr>
<tr>
<td>Smoking status (%)</td>
<td></td>
</tr>
<tr>
<td>Never smoked</td>
<td>34.3</td>
</tr>
<tr>
<td>Past smoker</td>
<td>33.0</td>
</tr>
<tr>
<td>Current smoker</td>
<td>32.7</td>
</tr>
<tr>
<td>Fasting at blood drawing (%)</td>
<td>56.2</td>
</tr>
<tr>
<td>Postmenopausal status (%)</td>
<td>90.8</td>
</tr>
<tr>
<td>Postmenopausal hormone use (%)</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>41.7</td>
</tr>
<tr>
<td>Current</td>
<td>39.9</td>
</tr>
<tr>
<td>Past</td>
<td>18.0</td>
</tr>
</tbody>
</table>

$^1$ NA, not applicable.

$^2$ $n$ is the number of participants who returned the 1990 questionnaire and had no missing values for the variables listed in the table.

$^3$ Wilcoxon’s rank-sum test for variables expressed as $\bar{x} \pm$ SD and Pearson’s chi-square test for variables expressed as percentages.

$^4$ $\bar{x} \pm$ SD (all such values).
average intake from 1984 to 1990 and the biomarker concentrations (Table 4). We restricted our analysis to PUFA s and trans fatty acids, for which reasonable correlation coefficients were observed in Table 3. For most PUFA s, biomarkers were most strongly correlated with intake measured in 1990 when blood samples were collected. The correlations for intake measured in 1984 were mildly attenuated. For example, the correlation coefficients for intake measured in 1990 when blood samples were collected. The correlations for intake measured in 1990 were not different from, or even stronger than, those for intakes from the 1990 FFQ. In particular, modestly improved correlations were seen for EPA, linoleic acid, and trans fatty acids.

Linear trends of fatty acids in plasma and erythrocytes across deciles of average dietary fat from 1984 to 1990 (percentage of total fat intake) are shown in Figure 1. After adjustment for total energy and other covariates, clear dose-response relations were observed between DHA, linoleic acid, and trans fatty acids in plasma and erythrocytes and their intakes. P values for linear trend were all <0.001. Linoleic acid concentrations in plasma and erythrocytes plateaued at a high intake; this may partly explain the lower correlations observed for EPA, linoleic acid, and trans fatty acids.

Spearman’s correlation coefficients between plasma and erythrocyte fatty acids are shown in Figure 2. For each fatty acid or fatty acid group, the plasma content was closely correlated with the erythrocyte content, especially for the fatty acids that are largely exogenously produced. The average correlation coefficient was 0.72. Within plasma and erythrocytes, the fatty acids that accounted for a high proportion of total fatty acids were inversely correlated with each other (data not shown); trans 18:1

### Table 2

Fatty acid composition in plasma and erythrocytes and intake of fatty acids measured with a food-frequency questionnaire at baseline: the Nurses’ Health Study, 1990

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Plasma</th>
<th>Erythrocytes</th>
<th>Total fat</th>
<th>Total energy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Saturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:0</td>
<td>0.02 ± 0.03</td>
<td>0.00 ± 0.01</td>
<td>0.67 ± 0.45</td>
<td>0.21 ± 0.15</td>
</tr>
<tr>
<td>14:0</td>
<td>0.58 ± 0.28</td>
<td>0.19 ± 0.10</td>
<td>3.98 ± 1.37</td>
<td>1.27 ± 0.56</td>
</tr>
<tr>
<td>16:0</td>
<td>19.31 ± 2.41</td>
<td>18.65 ± 1.86</td>
<td>21.60 ± 2.58</td>
<td>6.87 ± 1.78</td>
</tr>
<tr>
<td>18:0</td>
<td>7.29 ± 0.78</td>
<td>13.14 ± 1.00</td>
<td>7.88 ± 1.56</td>
<td>2.53 ± 0.84</td>
</tr>
<tr>
<td>Total</td>
<td>28.71 ± 2.38</td>
<td>34.37 ± 1.60</td>
<td>33.74 ± 4.48</td>
<td>10.75 ± 2.87</td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:1(n-7)</td>
<td>1.94 ± 0.84</td>
<td>0.49 ± 0.26</td>
<td>1.94 ± 0.43</td>
<td>0.62 ± 0.20</td>
</tr>
<tr>
<td>18:1(n-9)</td>
<td>18.60 ± 2.43</td>
<td>13.26 ± 1.17</td>
<td>29.21 ± 3.08</td>
<td>9.29 ± 2.31</td>
</tr>
<tr>
<td>20:1(n-12)</td>
<td>0.02 ± 0.01</td>
<td>0.003 ± 0.01</td>
<td>0.29 ± 0.15</td>
<td>0.09 ± 0.05</td>
</tr>
<tr>
<td>Total</td>
<td>22.86 ± 3.01</td>
<td>18.64 ± 1.24</td>
<td>38.33 ± 2.70</td>
<td>12.18 ± 2.76</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-3</td>
<td>0.50 ± 0.15</td>
<td>0.18 ± 0.05</td>
<td>1.66 ± 0.54</td>
<td>0.52 ± 0.18</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.49 ± 0.21</td>
<td>1.15 ± 0.91</td>
<td>0.12 ± 0.11</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.44 ± 0.09</td>
<td>1.85 ± 0.28</td>
<td>0.04 ± 0.03</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>1.56 ± 0.60</td>
<td>3.71 ± 1.09</td>
<td>0.29 ± 0.22</td>
<td>0.08 ± 0.06</td>
</tr>
<tr>
<td>Total</td>
<td>2.94 ± 0.75</td>
<td>7.06 ± 1.90</td>
<td>2.11 ± 0.69</td>
<td>0.65 ± 0.20</td>
</tr>
<tr>
<td>n-6</td>
<td>30.58 ± 4.33</td>
<td>13.66 ± 1.80</td>
<td>16.54 ± 4.20</td>
<td>5.21 ± 1.64</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>7.80 ± 1.62</td>
<td>14.63 ± 1.29</td>
<td>0.23 ± 0.09</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>Total</td>
<td>41.16 ± 4.60</td>
<td>33.91 ± 1.79</td>
<td>16.79 ± 4.21</td>
<td>5.29 ± 1.64</td>
</tr>
<tr>
<td>Total polyunsaturated fatty acids</td>
<td>44.87 ± 4.81</td>
<td>43.48 ± 1.77</td>
<td>19.03 ± 4.12</td>
<td>5.97 ± 1.55</td>
</tr>
<tr>
<td>trans Fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9c,12c-18:2n-6</td>
<td>0.10 ± 0.05</td>
<td>0.16 ± 0.35</td>
<td>3.71 ± 1.27</td>
<td>1.18 ± 0.47</td>
</tr>
<tr>
<td>9c,12c-18:3n-3</td>
<td>0.26 ± 0.10</td>
<td>0.12 ± 0.05</td>
<td>0.25 ± 0.08</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>Total</td>
<td>0.72 ± 0.24</td>
<td>0.36 ± 0.10</td>
<td>0.69 ± 0.23</td>
<td>0.22 ± 0.09</td>
</tr>
<tr>
<td>Total trans fatty acids</td>
<td>1.98 ± 0.71</td>
<td>1.65 ± 0.43</td>
<td>4.67 ± 1.42</td>
<td>1.48 ± 0.54</td>
</tr>
</tbody>
</table>

1 All values are x ± SD; n = 306 unless otherwise indicated. Only fatty acids for which both composition in plasma and erythrocytes and dietary assessments are available are reported.
2 n = 130 for plasma and 132 for erythrocytes.
3 t denotes trans configuration; c, cis configuration.
TABLE 3
Spearman’s correlation coefficients between fatty acid composition in plasma and erythrocytes and intake measured with the 1990 food-frequency questionnaire: the Nurses’ Health Study, 1990

| Fatty acids                  | Plasma Unadjusted | Plasma Adjusted | Erythrocytes Unadjusted | Erythrocytes Adjusted | P
|------------------------------|------------------|-----------------|-------------------------|-----------------------|---
| Saturated fatty acids        |                  |                 |                         |                       |   
| 12:0                         | 0.14             | 0.16            | 0.12                    | 0.13                  | NS |
| 14:0                         | 0.20             | 0.20            | 0.17                    | 0.16                  | NS |
| 16:0                         | 0.15             | 0.12            | 0.08                    | 0.03                  | NS |
| 18:0                         | 0.05             | 0.06            | –0.03                   | 0.01                  | NS |
| Total                        | 0.20             | 0.16            | 0.15                    | 0.12                  | NS |
| Monounsaturated fatty acids   |                  |                 |                         |                       |   
| 16:1n–7                      | 0.16             | 0.16            | 0.15                    | 0.10                  | NS |
| 18:1n–9                      | 0.10             | 0.12            | 0.12                    | 0.14                  | NS |
| Total                        | 0.04             | 0.04            | 0.06                    | 0.05                  | NS |
| Polyunsaturated fatty acids   |                  |                 |                         |                       |   
| n–3                          |                  |                 |                         |                       |   
| 18:3n–3                      | 0.23             | 0.23            | 0.17                    | 0.18                  | NS |
| 20:5n–3                      | 0.27             | 0.21            | 0.23                    | 0.38                  | < 0.01 |
| 22:5n–3                      | 0.01             | −0.03           | 0.02                    | 0.01                  | NS |
| 22:6n–3                      | 0.47             | 0.48            | 0.54                    | 0.56                  | < 0.01 |
| Total                        | 0.31             | 0.30            | 0.42                    | 0.41                  | < 0.05 |
| n–6                          |                  |                 |                         |                       |   
| 18:2n–6                      | 0.24             | 0.25            | 0.19                    | 0.24                  | NS |
| 20:4n–6                      | 0.003            | −0.01           | −0.06                   | −0.04                 | NS |
| Total                        | 0.21             | 0.21            | 0.16                    | 0.19                  | NS |
| Total polyunsaturated fatty acids |      |                 |                         |                       |   
| trans Fatty acids            |                  |                 |                         |                       |   
| 16:1n–7                      | 0.26             | 0.27            | 0.23                    | 0.25                  | NS |
| Total 18:1 trans isomers     | 0.27             | 0.29            | 0.39                    | 0.42                  | < 0.001 |
| 9t,12t–18:2n–6               | 0.26             | 0.25            | 0.24                    | 0.26                  | NS |
| 9c,12r–18:2n–6               | 0.17             | 0.15            | 0.20                    | 0.19                  | NS |
| Total 18:2 trans isomers     | 0.21             | 0.19            | 0.25                    | 0.23                  | NS |
| Total trans fatty acids      | 0.29             | 0.30            | 0.41                    | 0.43                  | < 0.01 |

1 n = 306 unless otherwise indicated.
2 Spearman’s correlation coefficients were adjusted for age at blood drawing, BMI, current weight, smoking status, postmenopausal status, postmenopausal hormone use, period of blood assay, and fasting status at blood drawing. P < 0.05 for coefficients ≥ 0.09 and P < 0.01 for coefficients ≥ 0.10 (t test) unless otherwise indicated.
3 Wolfe’s test for one-sample comparison of Spearman’s partial correlation coefficients between plasma and erythrocytes.
4 n = 130 for plasma (P < 0.05 for coefficients ≥ 0.14 and P < 0.01 for coefficients ≥ 0.16); n = 132 for erythrocytes (P < 0.05 for coefficients ≥ 0.14 and P < 0.01 for coefficients ≥ 0.15).
5 t test configuration; c, cis configuration.

isomers were strongly positively correlated with trans 18:2 isomers in both plasma (r = 0.77) and erythrocytes (r = 0.66).

DISCUSSION

We observed moderate-to-strong correlations between n–3 fatty acids of marine origin and trans fatty acids in erythrocytes and plasma and corresponding intakes measured with validated FFQs; overall, correlations with intake were stronger for erythrocytes than for plasma. Our study population did not differ from the whole Nurses’ Health Study cohort with respect to age, BMI, diet, and other characteristics that could influence the correlations between biomarkers and intake. Although the study participants were older and more likely to be smokers and postmenopausal, these characteristics did not substantially alter the correlations in the analysis.

As expected, saturated fatty acids and MUFAs in plasma and erythrocytes did not reflect intake, probably because these 2 classes of fatty acids could be endogenously synthesized from carbohydrates. In line with previous studies (20–25, 37), n–3 fatty acids of marine origin and trans fatty acids in plasma or erythrocytes provided the strongest correlations with intake. In general, erythrocyte fatty acids were more strongly correlated with intake than were plasma fatty acids. For example, correlation coefficients of 0.23–0.55 were observed for EPA in plasma fractions (20, 21, 37, 38), whereas relatively higher correlation coefficients (0.36–0.58) were observed for EPA in erythrocytes (22–25). However, it is complicated to make direct comparisons between plasma and erythrocytes on the basis of these data, because the dietary assessment methods used, population characteristics, and true between-person variation of intake and biomarker concentrations can be quite different among these studies. All of these factors could influence the correlation coefficients; therefore, any differences between plasma and erythrocytes could result from either these factors or the inherent differences between these 2 specimens (3, 8).
To our knowledge, the current study is the first to examine the performance of plasma and erythrocytes to reflect intake measured with FFQs within the same population. The half-life of erythrocytes is 120 d, which is much longer than that of plasma lipoproteins. Erythrocytes are therefore hypothesized to be a more appropriate specimen to reflect long-term intake than plasma or serum. In one controlled dietary trial, Katan et al (19) clearly showed that serum cholesteryl esters were more sensitive to recent diet than were erythrocytes. In serum cholesteryl esters the incorporation half-life of EPA was 4.8 d, and the concentration of EPA peaked after 1–2 mo of fish-oil supplementation. In contrast, in erythrocytes the incorporation half-life of EPA was 4 wk, and the concentration of EPA plateaued at 6 mo of supplementation. The authors concluded that serum may reflect intake over the past weeks, whereas erythrocytes reflect intake over the past months (19). This is the likely explanation for our findings of higher correlations for n–3 fatty acids of marine origin and trans fatty acids in erythrocytes than in plasma.

The half-life of fatty acids in adipose tissue was estimated to be 680 d (16), which suggested that adipose tissue better reflected the long-term intake of fatty acids than did other specimens. One controlled dietary trial showed evidence supporting this hypothesis (19). Similarly, Baylin et al (20) showed stronger correlations for n–6 fatty acids, linolenic acid, and trans fatty acids in adipose tissue than in plasma or whole blood (20). In a Norwegian population whose intake of n–3 fatty acids of marine origin was high, total serum reflected long-term intake as strongly as did adipose tissue (8). In another study, the n–3 fatty acid concentration in erythrocytes was even better correlated with intake than was the n–3 fatty acid concentration in adipose tissue (25). The

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**TABLE 4**

Spearman’s partial correlation coefficients between fatty acid composition in plasma and erythrocytes and intake measured with a food-frequency questionnaire (FFQ) during various periods: the Nurses’ Health Study, 1984–1990.

<table>
<thead>
<tr>
<th>Intake by period</th>
<th>Plasma</th>
<th>Erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:5n–3</td>
<td>0.34</td>
<td>0.30</td>
</tr>
<tr>
<td>20:6</td>
<td>0.17</td>
<td>0.37</td>
</tr>
<tr>
<td>18:2n–6</td>
<td>0.21</td>
<td>0.38</td>
</tr>
<tr>
<td>Cumulative average</td>
<td>0.28</td>
<td>0.41</td>
</tr>
<tr>
<td>22:6n–3</td>
<td>0.33</td>
<td>0.41</td>
</tr>
<tr>
<td>18:2n–6</td>
<td>0.36</td>
<td>0.43</td>
</tr>
<tr>
<td>1990</td>
<td>0.48</td>
<td>0.56</td>
</tr>
<tr>
<td>Cumulative average</td>
<td>0.44</td>
<td>0.54</td>
</tr>
<tr>
<td>18:2n–6</td>
<td>0.17</td>
<td>0.16</td>
</tr>
<tr>
<td>1990</td>
<td>0.25</td>
<td>0.24</td>
</tr>
<tr>
<td>Cumulative average</td>
<td>0.32</td>
<td>0.27</td>
</tr>
<tr>
<td>Total trans fatty acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1984</td>
<td>0.28</td>
<td>0.38</td>
</tr>
<tr>
<td>1990</td>
<td>0.31</td>
<td>0.34</td>
</tr>
<tr>
<td>1990</td>
<td>0.30</td>
<td>0.43</td>
</tr>
<tr>
<td>Cumulative average</td>
<td>0.37</td>
<td>0.48</td>
</tr>
</tbody>
</table>

1 Spearman’s correlation coefficients were adjusted for age at blood drawing, BMI, current weight, smoking status, postmenopausal status, postmenopausal hormone use, period of blood assay, and fasting status at blood drawing. P < 0.05 for coefficients ≥ 0.09 and P < 0.01 for coefficients ≥ 0.10 (t test).

2 1984 denotes intake measured by 1984 FFQ (n = 289); 1986, intake measured by 1986 FFQ (n = 273); 1990, intake measured by 1990 FFQ (n = 306).

3 Plasma: n = 111 for 1984, n = 115 for 1986, and n = 130 for 1990 and cumulative average intake. Erythrocytes: n = 113 for 1984, n = 117 for 1986, and n = 132 for 1990 and cumulative average intake. P < 0.05 for coefficients ≥ 0.15 and P < 0.01 for coefficients ≥ 0.17.

4 Average intake calculated from all 3 FFQs (n = 306).
much lower concentrations of n−3 fatty acids in adipose tissue in this population may have resulted in less precise measurements and may partly explain this finding (25). In a group of Nurses’ Health Study participants, Garland et al (17) used the same FFQs that were used in the present study to compare fatty acids in adipose tissue with fatty acid intake. The correlations for trans fatty acids reported in that study (r = 0.43 for 18:1 trans isomers, r = 0.22 for 18:2 trans isomers, and r = 0.40 for total trans fatty acids) were not significantly different from the correlations of trans fatty acids in erythrocytes in the current study. In another study conducted in women living in the Boston, Massachusetts, area who had characteristics similar to our study participants, London et al (30) showed a correlation coefficient of 0.48 for n−3 fatty acids of marine origin (EPA plus DHA) in adipose tissue and intake derived from the same FFQs. This correlation was not different from the correlations for EPA and DHA in erythrocytes observed in our study (rEPA = 0.38 for EPA and rDHA = 0.56 for DHA). These observations indicated that erythrocytes might reflect the long-term intake of n−3 fatty acids of marine origin and of trans fatty acids to an extent comparable with that of adipose tissue. In consideration of the limited availability of fat aspirate samples in epidemiologic studies, erythrocytes may warrant more attention as a medium to reflect long-term fatty acid intake.

The PUFAs in plasma and erythrocytes tended to be most strongly correlated with intake estimated by the 1990 FFQ. The correlations were slightly weakened when intake was estimated by the 1984 FFQ. In the current study the FFQs inquired about intake over the previous year. If the assumption were true that intake of fatty acids is constant over time, we should have observed similar correlations over the previous 6 y. The mild gradient of correlations we observed over time may have been due to changes in dietary intake over time. In general, intrapersonal variation in intake weakens the correlation coefficients between biomarkers and diet. One way to minimize this type of measurement error is to obtain long-term average intakes from repeated dietary measurements over previous years. In our study, the average intakes of polyunsaturated and trans fatty acids calculated from the 1984, 1986, and 1990 FFQs correlated better with biomarker concentrations than did those calculated from the 1990 FFQ alone. In a prospective analysis of dietary fatty acids and coronary heart disease risk, we showed that the analyses using repeated measures of diet yielded stronger associations than did those using only baseline diet or most recent diet (39).

Perfect correlations between fatty acids in tissues and intakes measured by FFQs are unrealistic. Persons with the same fatty acid intake may not have the same concentration of that fatty acid in tissues (4, 40). Nondietary factors, such as absorption, metabolism, and genetic and lifestyle determinants, can affect fatty acid concentrations in human tissues (1). In addition to the differences in fatty acid composition in blood fractions (plasma and erythrocytes) and dietary fat, we also observed considerable differences between these 2 blood fractions. These differences may have been due to the endogenous synthesis of some fatty acids, the different physiologic functions of certain fatty acids in different blood fractions, or different roles of these fractions as vehicles for fatty acid transport (1). However, because measurement errors of the biomarkers and the FFQs are independent, the moderate-to-strong correlation coefficients observed in the current study support the relative ability of biomarkers to reflect usual fatty acid intake.

In summary, we observed moderate-to-strong correlations between n−3 fatty acids of marine origin and trans fatty acids in erythrocytes and corresponding intakes, especially when multiple FFQs in preceding years were used to calculate long-term usual dietary intakes. Correlations with intake were generally stronger for erythrocytes than for plasma, which suggests that erythrocytes may be considered as an alternative to adipose tissue for these measurements.

We are indebted to Frank Sacks and Walter Willett for their valuable comments and for editing this manuscript and to Bernard Rosner for providing critical statistical guidance.

The authors’ responsibilities were as follows—QS: analyzed the data and drafted the manuscript; JM and SEH: designed the study and directed the blood sample assays; HC: designed the study, assayed the biomarkers, and prepared the data; FBH: designed the study’s analytic strategy and supervised the data analysis; and all authors: contributed to the revision of the manuscript. None of the authors had any financial or personal conflict of interest to disclose.
Scaling of human body composition to stature: new insights into body mass index

Steven B Heymsfield, Dympna Gallagher, Laurel Mayer, Joel Beetsch, and Angelo Pietrobelli

ABSTRACT
Background: Although Quetelet first reported in 1835 that adult weight scales to the square of stature, limited or no information is available on how anatomical body compartments, including adipose tissue (AT), scale to height.

Objective: We examined the critical underlying assumptions of adiposity–body mass index (BMI) relations and extended these analyses to major anatomical compartments: skeletal muscle (SM), bone, residual mass, weight (AT+SM+bone), AT-free mass, and organs (liver, brain).

Design: This was a cross-sectional analysis of 2 body-composition databases: one including magnetic resonance imaging and dual-energy X-ray absorptiometry (DXA) estimates of evaluated components in adults (total n = 411; organs = 76) and the other a larger DXA database (n = 1346) that included related estimates of fat, fat-free mass, and bone mineral mass.

Results: Weight, primary lean components (SM, residual mass, AT-free mass, and fat-free mass), and liver scaled to height with powers of ≈2 (all P < 0.001); bone and bone mineral mass scaled to height with powers >2 (2.31–2.48), and the fraction of weight as bone mineral mass was significantly (P < 0.001) correlated with height in women. AT scaled weakly to height with powers of ≈2, and adiposity was independent of height. Brain mass scaled to height with a power of 0.83 (P = 0.04) in men and nonsignificantly in women; the fraction of weight as brain was inversely related to height in women (P = 0.002).

Conclusions: These observations suggest that short and tall subjects with equivalent BMIs have similar but not identical body composition, provide new insights into earlier BMI-related observations and thus establish a foundation for height-normalized indexes, and create an analytic framework for future studies.


KEY WORDS Height, brain mass, liver mass, skeletal muscle, adipose tissue, obesity

INTRODUCTION
Quetelet, in his 1835 classic treatise Sur l’homme et le développement de se facultés, ou Essai de physique sociale, first made the observation that weight increases in adults of normal build in proportion to the square of height (1, 2). More than a century later, Keys and his colleagues, while evaluating weight-height indexes as measures of adiposity, identified Quetelet’s index (weight/height2) as having the highest correlations with skinfold and body density measurements (3). Body mass index (BMI), as Keys referred to weight/height2, has since been extensively studied as a phenotypic marker of adiposity in children and adults (4–17).

The classic studies of Quetelet (1, 2) and Keys et al (3) provide the foundation for applying BMI as an optimum weight-height index of adiposity. The evolved concepts include 3 main mathematical constructs, the first of which is that weight scales to height2 and thus weight/height2 (ie, BMI) is independent of height (6, 11). Several early studies support the validity of this assumption (11), although there is some variability in the exponent or power of height above and below 2 (11–13, 15, 18). Others argued on the basis of the work of Benn (6) that the power of height should be population specific (13, 14), and this suggestion was incorporated into what became known as the Benn Index (weight/heightβ, with β population specific). The sources of variation in observed powers when weight is scaled to height remain unknown.

A second related tenet of the BMI model, although one rarely studied, is that adipose tissue also scales to the square of height. Unless weight and adipose tissue scale to the same power of height, short and tall subjects will differ in adiposity. The question of the stature dependency of adiposity has not been rigorously examined (6, 19), although Garrow and Webster (9) were unable to find a significant association between height and percentage fat in a small cohort of women.

A third and important feature of the BMI model is that adiposity, which is defined as adipose tissue mass/weight, is maximally correlated with weight/heightβ when β is equal to 2. Keys et al (3) and others (7, 12, 15) examined powers of height in addition to 2 and largely confirmed that 2 was the nearest integer providing maximal correlations with measures of adiposity.

1 From Merck & Company, Rahway, NJ (SBH and JB); the New York Obesity Research Center (DG) and the Department of Psychiatry (LM), Columbia University, College of Physicians and Surgeons, New York, NY; and the Pediatric Unit, Verona University Medical School, Verona, Italy (AP).
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Although adiposity is of central interest in relation to BMI, other compartments of importance also contribute to how weight scales to height. Moreover, there is an implicit but not well articulated assumption that subjects of the same BMI but who differ in height have the same relative amount of skeletal muscle, bone, and components other than adipose tissue. This theory states that short and tall subjects of equivalent BMI have identical fractional body composition (ie, component weight/body weight), not just adiposity. Accordingly, in the current study, we critically examined the scaling of anatomical body compartments to height with the aim of clarifying prevailing questions related to the now widely applied phenotypic measure, BMI.

SUBJECTS AND METHODS

Experimental design and rationale

Allometric models

Existing databases were used to examine 3 questions, the first 2 of which were: How do weight and major anatomical and closely related molecular-level body compartments scale to height in healthy adults? When expressed as a fraction of body mass, are these compartments independent of height? The scaling of weight and each component to height, used to examine these 2 questions, was evaluated by using the traditional allometric model

\[ y = \alpha \times x^\beta + \epsilon \]

where \( y \) is body weight or component mass, \( x \) is height, \( \beta \) is the scaling exponent or power, \( \alpha \) is the proportionality constant, and \( \epsilon \) is a multiplicative error term (11, 16, 17). When converted to logarithmic form, the allometric equation can be solved as

\[ \log_y y = \log_y \alpha + \beta \log_x x + \log_\epsilon \epsilon \]

According to Quetelet, \( \beta \) in Equations 1 and 2 for weight (\( y \)) scaled to height (\( x \)) is equal to 2. When weight (or component)/height\( ^\beta \) is plotted against height, the correlation will be non-significant when \( \beta \) is derived in the population under study as emphasized by Benn (6). If \( \beta \) is assumed to be 2 as in BMI and the actual value differs from 2, there exists the possibility of bias in adjusted weights or component mass. Dividing weight or component mass by height\( ^\beta \) when \( \beta \neq 2 \) may over- or under-correct for between-individual differences in stature. In addition to weight, there is growing interest in expressing body-composition results in the form of height-normalized indexes, ie, component/height\( ^\beta \) (20, 21), although there has been little examination of how these selected components actually scale to height.

In addition to Equation 2, the value of \( \beta \) and a related error term can also be estimated by using an approach suggested by Benn (6). Flegal (18) showed close agreement between the 2 methods and we also confirmed nearly identical results for both methods in the present study. Accordingly, we provide estimates for \( \beta \) in the results only for the log-log approach as stated in Equation 2.

A common practice is to express component mass as a fraction or percentage of weight. The background for the analysis of the second question is formulated on the ratio of component mass to weight, each of which scale individually to height:

\[ \text{Component} = \alpha_1 \text{height}^{\beta_1} \]  
\[ \text{Weight} = \alpha_2 \text{height}^{\beta_2} \]

Therefore,

\[ \text{Component/weight} = \frac{\alpha_1}{\alpha_2} \text{height}^{\beta_1-\beta_2} \]

When \( \beta_1 \) and \( \beta_2 \) are equal (ie, the component and body weight scale the same to height), the value of \( \beta \) is 0 and a non-zero number raised to the power of zero equals 1. Thus, there will be no association between component fractional mass and height if the difference between \( \beta_1 \) and \( \beta_2 \) is at or near zero. If the difference between \( \beta_1 \) and \( \beta_2 \) in Equation 5 is not zero, the fractional component mass will scale positively or negatively to height, possibly significantly. If a component and weight scale differently to height, short and tall subjects will not have the same body composition.

Maximal associations

What is the power (\( \beta \)) of height in the ratio weight/height\( ^\beta \) that maximizes the component’s correlation with adiposity and muscularity? Adiposity is generally defined as the amount of adipose tissue or fat present relative to body weight. A similar definition applies to muscularity. The basis of this third question is formulated on the studies of Keys et al (3), who were the first of many to examine the correlations between adiposity (adipose tissue or fat mass/weight) and weight/height\( ^\beta \), with \( \beta = 2 \) in BMI. In exploring this question, we established the optimum values of \( \beta \) for adiposity and muscularity because of their clinical and research relevance. The approach suggested by Benn (6) and Larson et al (12) was applied, in which \( R^2 \) values are generated for the regressions of component/weight on weight/height\( ^\beta \) for varying values of \( \beta \). The resulting data can then be used to plot \( R^2 \) (variance) values versus \( \beta \) for the component of interest, and visual inspection is then used to select the \( \beta \) value or values associated with the maximum correlation.

Subjects and measurements

The 3 questions were examined by using data from healthy normal-weight and obese adults of varying ethnicity collected across multiple studies at the New York Obesity Research Center. The first evaluated database (NY-1) included subjects with whole-body magnetic resonance imaging (MRI) (22, 23) and dual-energy X-ray absorptiometry (DXA) studies (22–24). The developed anatomical body-composition model for all subjects included 4 major body compartments: adipose tissue, skeletal muscle, bone, and residual mass. Residual mass, the difference between weight and the other 3 measured components, includes high-metabolic-rate tissues and organs such as liver, brain, heart, and kidneys (23). Bone mass was estimated from DXA-measured bone mineral mass (22, 23), and estimates of adipose tissue, skeletal muscle, and residual mass were provided by MRI (22, 23, 25).

In addition, a subset of the subjects evaluated in the NY-1 studies completed separate imaging procedures for estimation of 2 residual mass components, brain and liver, by use of the methods reported by Gallagher et al (23). Adipose-tissue-free mass (ATFM) was calculated as the difference between weight and adipose tissue mass. This group is referred to as the NY-1A subgroup of the larger NY-1 database in subsequent presentations.

The second database (NY-2) included healthy adult subjects evaluated solely by DXA for total body fat, fat-free mass (FFM), and bone mineral mass (8, 22–24). Fat and FFM as measured by
DXA are the molecular body-composition level counterparts of the anatomical level components adipose tissue and ATFM (22). Bone mass in the NY-1 group was also derived from DXA-measured bone mineral mass, and the 2 differed only by a constant (22, 23). Evaluation of the NY-2 database was prompted by findings in the smaller NY-1 database that suggested the appropriateness of confirming fat and bone mineral scaling to height in a much larger and more diverse database.

Both databases included subjects with a minimum BMI (in kg/m²) of 18.5 and a maximum BMI of 35; the latter cutoff was due to MRI and DXA size limitations. Subjects in all databases were aged > 18 y with no imposed maximum age. Body weight and height in all subjects were measured with a calibrated digital scale and stadiometer, respectively.

Statistical methods

Baseline subject demographic characteristics are reported as means and SDs. The statistical analyses were carried out by using SPSS (SPSS for WINDOWS, version 11.5; SPSS Inc, Chicago, IL).

The allometric model coefficients in Equation 2, \( \alpha \) and \( \beta \), were derived by using least-squares multiple linear regression analysis and log-transformed data with weight or component mass set as the dependent variable and height and potentially age as independent variables. Values for \( \log \alpha \) and \( \beta \) along with \( R \) and SEE values for each developed regression model are presented in the Results. Student’s \( t \) tests were used to compare values of \( \beta \) for the height predictor variable in regression models compared with the reference \( \beta \) value of 2.0. In expanded analyses, we also developed regression models with height alone as an independent variable within discrete age groups in the large NY-2 database. These results were concordant with the pooled group analyses and are not presented.

Review of data in the first series of allometric analyses indicated that 2 components consistently scaled to height differently from weight, namely, bone and brain. Accordingly, we specifically examined the correlations between bone/weight and brain/weight with height. These analyses were carried out by multiple regression analysis. None of the other component/weight associations with height were statistically significant.

We regressed the fractions of weight as adipose tissue and skeletal muscle against body weight/height \( \beta \) by using simple linear regression analysis or a second-order polynomial, depending on the established data structure. Values of \( \beta \) were systematically varied from 0 to 3 in increments of 0.5 following preliminary analyses. Benn (6) and Larsson et al’s (12) method was used to establish values of \( \beta \) that maximally correlated with adiposity and muscularity.

RESULTS

Baseline group characteristics

The baseline demographic information for the study groups is summarized in Table 1. The 2 groups, NY-1 and NY-2, collectively had 1757 subjects, 759 men and 998 women (NY-1, 411; NY-1A, 76; NY-2, 1346). The groups ranged in mean age from 39.4 to 49.8 y and in BMI from 24.3 to 26.1. The NY-1A subgroup included 76 subjects, 19 men and 57 women, who did not differ significantly in mean BMI from the main NY-1 group. The NY-1 and NY-1A men did not differ in age, whereas the NY-1 women were significantly older (\( P = 0.03 \)) than their NY-1A counterparts.

Allometric analyses

We present both the NY-1 and the NY-2 results in parallel, starting with weight–stature relations and then advancing to each of the evaluated body compartments. The regression model results presented in this section are summarized in Table 2 for the NY-1 and NY-2 groups. The associations of weight and each component with height, fitted with univariate regression models, are presented in Figure 1 and Figure 2 for NY-1 men and women, respectively. The corresponding scatter plots for NY-2 men and women are presented in Figure 3.

Body weight

Weight scaled with respective powers of height (± SEE) minimally below and above 2 in the NY-1 and NY-2 men (1.78 ± 0.25 and 1.86 ± 0.13) and women (2.17 ± 0.27 and 2.17 ± 0.14), with all 4 models highly significant (all \( P < 0.001 \)). None of these respective powers differed significantly from 2.0. Age was a significant positive predictor of weight, after control for height, in all 4 regression models. BMI (ie, weight/height²) and weight/height raised to the respective actual powers (ie, 1.78–2.17) were also independent of height in the male and female groups.

Lean compartments

Skeletal muscle mass

Skeletal muscle mass scaled to height with powers of 1.98 ± 0.27 and 2.08 ± 0.20 in the NY-1 men and women (Table 2; both

| Table 1 Subject characteristics of the NY-1 and NY-2 database groups\(^1\) |
|-----------------|-----------------|-----------------|-----------------|
|                 | NY-1            | NY-1A           | NY-2            |
|                 | Men (n = 178)   | Women (n = 233) | Men (n = 19)    | Women (n = 57) | Men (n = 581) | Women (n = 765) |
| Age (y)         | 49.8 ± 15.8     | 44.9 ± 17.4     | 48.9 ± 21.1     | 39.4 ± 18.2\(^2\) | 44.8 ± 7.7    | 47.9 ± 18.0     |
| Weight (kg)     | 80.6 ± 12.4     | 68.7 ± 15.0     | 78.1 ± 10.1     | 65.4 ± 16.0     | 76.8 ± 12.4   | 65.0 ± 11.5     |
| Height (cm)     | 1.77 ± 0.07     | 1.62 ± 0.07     | 1.79 ± 0.08     | 1.63 ± 0.07     | 1.74 ± 0.08   | 1.62 ± 0.07     |
| BMI (kg/m²)     | 25.7 ± 3.6      | 26.1 ± 5.4      | 24.3 ± 2.5      | 24.7 ± 5.5      | 25.3 ± 3.4    | 24.9 ± 14.1     |

\(^1\) All values are \( \bar{x} \) ± SD. The databases are described in Subjects and Methods.

\(^2\) Significantly different from the NY-1 females, \( P = 0.03 \). (There were no other significant differences between the NY-1 and NY-1A groups.)
TABLE 2

Body composition–stature associations of the NY-1 and NY-2 database groups

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th></th>
<th>Model 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ht</td>
<td>Age</td>
<td>SEE</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NY-1</td>
<td>1.78</td>
<td>0.07</td>
<td>−5.08</td>
<td>0.14</td>
</tr>
<tr>
<td>NY-2</td>
<td>1.86</td>
<td>0.11</td>
<td>−5.72</td>
<td>0.16</td>
</tr>
<tr>
<td>AT, NY-1 (kg)</td>
<td>1.76</td>
<td>0.35</td>
<td>−7.65</td>
<td>0.41</td>
</tr>
<tr>
<td>Fat, NY-2 (kg)</td>
<td>1.89</td>
<td>0.47</td>
<td>−8.39</td>
<td>0.38</td>
</tr>
<tr>
<td>ATFM, NY-1 (kg)</td>
<td>2.09</td>
<td>NS</td>
<td>−6.77</td>
<td>0.10</td>
</tr>
<tr>
<td>FFM, NY-2 (kg)</td>
<td>1.86</td>
<td>−0.04</td>
<td>−5.55</td>
<td>0.11</td>
</tr>
<tr>
<td>SM, NY-1 (kg)</td>
<td>1.98</td>
<td>−0.11</td>
<td>−6.33</td>
<td>0.14</td>
</tr>
<tr>
<td>Bone, NY-1 (kg)</td>
<td>2.42</td>
<td>−0.06</td>
<td>−9.88</td>
<td>0.12</td>
</tr>
<tr>
<td>Mo, NY-2 (kg)</td>
<td>2.31</td>
<td>−0.12</td>
<td>−10.50</td>
<td>0.13</td>
</tr>
<tr>
<td>RM, NY-1 (kg)</td>
<td>2.22</td>
<td>0.13</td>
<td>−9.10</td>
<td>0.14</td>
</tr>
<tr>
<td>Brain, NY-1 (kg)</td>
<td>0.83</td>
<td>NS</td>
<td>−3.83</td>
<td>0.07</td>
</tr>
<tr>
<td>Liver, NY-1 (kg)</td>
<td>2.65</td>
<td>NS</td>
<td>−13.2</td>
<td>0.15</td>
</tr>
<tr>
<td>Women</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NY-1</td>
<td>2.17</td>
<td>0.19</td>
<td>−7.52</td>
<td>0.18</td>
</tr>
<tr>
<td>NY-2</td>
<td>2.17</td>
<td>0.05</td>
<td>−7.06</td>
<td>0.13</td>
</tr>
<tr>
<td>AT, NY-1 (kg)</td>
<td>2.15</td>
<td>0.54</td>
<td>−9.89</td>
<td>0.41</td>
</tr>
<tr>
<td>Fat, NY-2 (kg)</td>
<td>2.58</td>
<td>0.63</td>
<td>−13.03</td>
<td>0.47</td>
</tr>
<tr>
<td>ATFM, NY-1 (kg)</td>
<td>2.20</td>
<td>NS</td>
<td>−7.45</td>
<td>−0.11</td>
</tr>
<tr>
<td>FFM, NY-2 (kg)</td>
<td>2.05</td>
<td>−0.08</td>
<td>−6.16</td>
<td>0.10</td>
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<tr>
<td>SM, NY-1 (kg)</td>
<td>2.08</td>
<td>NS</td>
<td>−7.55</td>
<td>0.14</td>
</tr>
<tr>
<td>Bone, NY-1 (kg)</td>
<td>2.48</td>
<td>−0.06</td>
<td>−10.26</td>
<td>0.12</td>
</tr>
<tr>
<td>Mo, NY-2 (kg)</td>
<td>2.38</td>
<td>−0.04</td>
<td>−11.03</td>
<td>0.12</td>
</tr>
<tr>
<td>RM, NY-1 (kg)</td>
<td>2.13</td>
<td>0.09</td>
<td>−8.56</td>
<td>0.17</td>
</tr>
<tr>
<td>Brain, NY-1 (kg)</td>
<td>0.001</td>
<td>NS</td>
<td>0.31</td>
<td>0.10</td>
</tr>
<tr>
<td>Liver, NY-1 (kg)</td>
<td>2.10</td>
<td>NS</td>
<td>−10.40</td>
<td>0.19</td>
</tr>
</tbody>
</table>

† The databases are described in Subjects and Methods. AT, adipose tissue; ATFM, adipose-tissue-free mass; FFM, fat-free mass; Ht, height; int, intercept; Mo, bone mineral mass; RM, residual mass; SM, skeletal muscle. Sample sizes for total AT, SM, bone, and RM: 178 males and 233 females in NY-1; sample sizes for brain and liver: 19 males and 57 females in NY-1A. Regression models were based on the allometric formula y = α · x^β, and are solved as log_y = log_α + β log_x + log_y. The regression models were developed by using SPSS (SPSS for Windows, version 11.5; SPSS Inc, Chicago, IL).

‡ Ht and Age are the respective values for β (slopes) in equation 2, whereas Int corresponds to log α.

§ ± SD (all such values).

¶ Power of height vs a power of 2.0: †P = 0.09, §P < 0.05. All other powers of height reported in the table were not significantly different from a power of 2.0; the power of height in the model for brain in women was not statistically significant.

oller mass

Bone mass

Bone mass scaled to height with powers of 2.42 ± 0.24 and 2.48 ± 0.17 in NY-1 men and women (Table 2), respectively. Bone mineral mass also scaled significantly to height in the NY-2 men and women (Table 2) with respective powers of 2.31 ± 0.12 and 2.38 ± 0.12. All 4 of the β values were significantly (P < 0.05) or borderline significantly (NY-1 men, P = 0.09) > 2.0. Age added significantly to the 4 regression models with negative coefficients in both men and women.

Among weight and all evaluated body compartments, the highest R values (0.65–0.70) tended to be for the bone-stature associations (Table 2). The values of β for bone scaled to height were consistently among the highest observed across weight and the multiple evaluated body compartments.

Residual mass

Residual mass scaled to height with powers of 2.22 ± 0.28 and 2.13 ± 0.28 in the NY-1 men and women (Table 2; both P = NS versus a power of 2.0), respectively. Age was a positive significant predictor of residual mass after control for height, and both models were highly significant in the men (R = 0.60, P < 0.001) and women (R = 0.48, P < 0.001).

The regression models for the 2 residual mass components, liver and brain, are summarized in Table 2, and scatter plots are presented in Figure 4. Liver mass scaled to height with powers of 2.65 ± 0.85 and 2.10 ± 0.61 in men and women (both P = NS versus a power of 2.0), respectively. The models were statistically significant in the men (R = 0.60, P = 0.005) and women (R = 0.43, P < 0.001) and did not include age as a covariate.

Unlike the other evaluated lean components, brain mass scaled weakly to height in the men (r = 0.46, P = 0.04) with a power of 0.83 ± 0.39 (P < 0.05 versus a power of 2.0) and nonsignificantly (r = 0.003, P = NS) in the women (Figure 4).

ATFM and FFM

The composite lean compartment, ATFM, scaled to height with powers of 2.09 ± 0.19 and 2.20 ± 0.17 in the NY-1 men and women (Table 2), respectively. Neither model, which were both statistically significant, included age as a predictor variable. The similar compartment, FFM, scaled to height with respective powers of 1.86 ± 0.10 and 2.05 ± 0.09 in the NY-2 men and women. Age was a small-magnitude, but statistically significant predictor variable in both models. All ATFM and FFM models were highly significant (P < 0.001), with R values ranging from 0.64 to 0.69; none of the β values differed significantly from 2.0.

Adipose tissue

Adipose tissue scaled to height with a power of 1.76 ± 0.75 in the NY-1 men and 2.15 ± 0.60 in the women with age a significant covariate in both models (Table 2). Fat mass also scaled significantly to height in the NY-2 men and women with respective powers of 1.86 ± 0.47 and 2.17 ± 0.33, but only after adding age to the models (Table 2). The allometric fat mass models were the only nonsignificant univariate correlations observed across the NY-2 group, as presented in Figure 3 for male and female subjects.

The 4 AT and fat mass models of the NY-1 and NY-2 groups had the lowest R values and largest SEEs among the allometric
models for components evaluated in the 2 respective groups. None of the observed $\beta$ values for height in the AT and fat prediction models differed significantly from 2.0.

**Stature-dependence of fractional mass**

Inspection of the univariate plots in Figures 1-3 and Table 2 indicate that most components scaled to height with powers similar to that of weight. Thus, according to Equation 5, their fractional mass will not correlate significantly with height. The association between liver/weight and height was not statistically significant in the NY-1A men even though liver scaled to height with a power of 2.65. After an initial screen of the remaining data, only bone and brain justified further analyses.

The fractions of weight as bone and bone mineral mass were not significantly correlated with height in either group of men. The association between fractional bone mass and height in the NY-1 women was borderline significant alone ($P = 0.07$), but significance was no longer present when age was added to the regression model. The fraction of weight as bone mineral was significantly correlated with height in the NY-2 women, even after adding age and age$^2$ as predictor variables in a multiple regression analysis model (model $R^2 = 0.36$; height covariate, $P < 0.001$). The fraction of weight as brain showed a trend or was significantly inversely correlated with stature in the men ($P = 0.07$) and women ($P = 0.002$), respectively (Figure 4).

**Maximal correlations**

The associations between component/weight and weight/height$^\beta$ were systematically examined for adipose tissue and skeletal muscle mass. The maximal correlations, expressed as $R^2$, between adipose tissue or fat/weight with weight/height$^\beta$ and the corresponding plots for skeletal muscle (Figure 5) were observed for values of $\beta$ in the range of 2–2.5. These ranges of maximal correlation were similar in men and women. These results are similar to those of Benn (6), who suggested the relatively flat ranges comprising peak correlations indicated only small differences in the value of selecting indexes with powers just above or below 2.
The present study is one of the first comprehensive examinations of anatomical body-composition scaling to stature in adults. Our observations strongly support those of Quetelet (1, 2) and many others (3–19, 26) that weight scales approximately to stature squared. We extended these classic observations by showing that other components, with the exception of brain and bone, also scale to height with powers approximating 2 as the nearest integer. Moreover, we confirmed (12) and extended earlier observations by showing that maximal correlations between adiposity and muscularity (ie, component mass/weight) and weight/height are present when height has values of 2–2.5. After appropriately adjusting for height, including adjustment as weight/height^2, the short and tall subjects in our sample had similar anatomical body composition, except for brain mass and to a lesser extent bone mass.

Although our samples were relatively small, as noted, we detected 2 “deviations” from the “y = x^2” rule. First, we observed consistently higher β values that differed significantly from 2.0 for the scaling of bone to height compared with the other evaluated components. A significantly higher fraction of weight as bone in tall subjects was, however, only observed in the relatively large NY-2 female sample. Tall subjects are heavier than short subjects because weight increases as height^2. Bone scales to weight in mammals with β values of 1.05–1.1, and Galileo was the first of many to draw attention to the relations between mammalian body size and bone structure/mass (27). Galileo advanced the concept that maintenance of the same relative bone strength, resistance to elastic buckling, bending, and torsion across animals differing by orders of magnitude in body mass requires proportionally thicker bones. The observation that mammalian skeletal mass scales to weight with powers greater than one is seen as consistent with this theory (28). Weight and bone in our subjects respectively scaled to height^-2 and height^-2.3, a small difference but one that is consistent with observations in mammals as a whole.

**FIGURE 2.** Weight and body composition versus height of women in the NY-1 database group. The plotted data were fit with univariate power functions (SPSS for WINDOWS, version 11.5; SPSS Inc, Chicago, IL) that are provided in each panel of the figure. The models are of the form, y = α x^β ε, where y is body weight or component mass, x is height, β is the scaling exponent or power, α is the proportionality constant, and ε is a multiplicative error term. All regression models in the figure are P < 0.05; the regression models including age as a covariate in addition to height are presented in Table 2. AT, adipose tissue; ATFM, adipose-tissue-free mass; SM, skeletal muscle mass; RM, residual mass.
Second, we observed that brain mass scales to height with values substantially and, at least in the women, the fraction of body weight as brain was significantly inversely related to stature. Cadaver studies generally report absent or small but statistically significant correlations between autopsy brain weight and height (29–31). Recent imaging studies, including those by Koh et al (32), Nopoulos et al (33), Chung et al (34), and others, largely support the cadaver studies with overall nonsignificant or weak correlations observed between brain volume and height. The reviewed earlier studies are thus consistent with our findings, which sharply contrast the relations between height and organ-tissue mass for brain compared with skeletal muscle, bone, and organs such as liver.

Our study results relate to a large anthropology and evolutionary biology literature that examines the relations between body size, brain mass, and performance, both mental and physical (28, 35–39). Achieving greater body size without compromising muscularity and not being encumbered by the greater energy demands of a proportionally increased brain mass must be a critical adaptive feature of Homo sapiens. Because brain has a very high mass-specific metabolic rate (ie, 240 kcal/kg versus 4.5 kcal/kg in adipose tissue), the possibility exists that mass-specific energy requirements (ie, kcal/kg weight) are lower in tall subjects than in their shorter counterparts.

Functional model of Quetelet’s index

The empirical findings of the present study suggest an anatomical and related functional basis for Quetelet’s index, which is now referred to as BMI. If stature is viewed as a dynamic process with “growth” from a small to large height, there is an essential requirement for greater structural and related functional support, including bone and skeletal muscle. We observed that bone and skeletal muscle scaled consistently to height with high R values, low SEE, and with powers at or very near 2. This lean tissue growth with increasing stature would require a similar expansion of metabolically supportive tissues encompassed by residual mass and its related components, such as liver. Our model, assembled from the observed study results, thus suggests that structural and metabolic lean tissue compartments grow in unison with greater stature. This hypothesis is consistent with the observation that both ATFM and FFM also scaled approximately as stature squared.

Unlike the major lean tissues, adipose tissue scaled less consistently to height with high SEE and low R values. This observation is consistent with the plasticity of adipose tissue as an energy storage compartment that can vary widely in mass with nutritional status, thus potentially obscuring associations with stature. Greater height with larger body size and metabolic
requirements would optimally include an appropriate subcutaneous adipose tissue insulation layer and energy stores, and this hypothesis provides one explanation for why adipose tissue and fat mass might in theory scale to height with powers similar to those of lean tissues.

The observations of the present study thus suggest the existence of a lean tissue “core” that scales consistently and strongly to height with a power not significantly different from 2.0. The corresponding associations for adipose tissue and fat mass are weaker, although values also did not differ significantly from 2.0. Thus, small differences between populations in adiposity, muscularity, and potentially secular effects may combine to introduce some variability in $\beta$ values around a mean of 2 for weight scaled to height. More focused studies including appropriate methods in specific populations may elucidate the basis for racial and geographic differences in the relations between adiposity and BMI.

Clinical applications

The results of the present study provide support for the suggestion of Van Itallie et al (20) to normalize fat and FFM for height$^2$ as a means of adjusting the mass of these components for between-subject differences in stature. Our findings, with the aforementioned provisos, suggest that skeletal muscle, bone, ATFM, FFM, and potentially liver mass also scale approximately as the square of height. Pending the study of larger and more representative samples, it thus seems reasonable that creating indexes of these and related components to height$^2$ would allow for body-composition comparisons between subjects or groups differing in height. This approach has been proposed for left ventricular mass measured by echocardiography, which appears to scale in adults as height$^{2.13}$ (40), which is similar to our observations for other nonneural lean tissues.

Study limitations

Although our sample of subjects evaluated with MRI overall was large given the expense and complexity of whole-body studies, we still lacked adequate power to detect small differences in component scaling to height. To some extent, our larger NY-2 subject database compensated for this limitation with lower SEEs for major component $\beta$ estimates (6). Our sample for brain and liver mass estimates was even smaller, which highlights the need to extend our exploratory observations to larger populations, particularly to groups differing in age and race (41). We did not consider more advanced questions, such as the effect on scaling of relative leg length and other skeletal proportions. Our study was limited to adults with BMIs between 18.5 and 35, and consideration should be given to subjects outside of this range. Advancing our studies to children and adolescents would also provide new insights into Quetelet’s rule when applied to persons other than adults (42). Quetelet noted that his height$^2$ rule was generally applicable across the life span, except during the first year of life and the pubertal period (1).
Conclusions

The results of the present study suggest that weight, skeletal muscle, adipose tissue, and bone mass/height are regressed against height with powers of 2 as the nearest whole integer. These observations indicate that corresponding height-normalized indexes are also independent of height, as are both adiposity and muscularity. Moreover, adiposity and muscularity maximally correlate with weight/height when β is = 2. These observations provide strong support for the application of BMI and height-normalized body composition indexes as stature-independent measures of relative adipose tissue and skeletal muscle mass.

The observations were less clear for bone, which scaled to height with powers minimally but consistently higher than those observed for weight scaled to height. Taller subjects, notably women, thus have similar but not identical body composition. These collective observations have broad-reaching implications for the study of human biology and to the clinical application of BMI as a surrogate measure of human body composition.

The authors’ contributions were as follows—SBH: principal investigator, design, data collection, analysis, and article preparation; DG: design, subject evaluation, and data collection; LM: design, subject evaluation, data collection, analysis, and article preparation; JB: design, analysis, and article preparation; AP: design, analysis, and article preparation. None of the authors had any financial or personal conflicts of interest.

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Treatment of anorexia nervosa is associated with increases in bone mineral density, and recovery is a biphasic process involving both nutrition and return of menses1–3


ABSTRACT

Background: Recovery from osteoporosis in anorexia nervosa (AN) is uncertain.

Objective: The purpose of this study was to understand the changes in bone mineral density (BMD) in women with AN and the mechanisms of recovery from osteopenia.

Design: We studied BMD and markers of bone formation and resorption, osteocalcin and N-telopeptide (NTX), in patients with AN (n = 28) who were following a behavioral weight-gain protocol.

Results: Anorexic patients experienced significant percentage increases in BMD (4.38 ± 7.48% for spine; 3.77 ± 8.8% for hip; P < 0.05 for both) from admission until recovery of 90% ideal body weight, achieved over 2.2 mo. NTX concentrations were higher in patients with AN at admission than in healthy control subjects (n = 11; 69.0 ± 31.09 and 48.3 ± 14.38 nmol/mmol creatinine, respectively; P < 0.05) and in reference control subjects (n = 30; 69.0 ± 31.09 and 37.0 ± 6.00 nmol/mmol creatinine, respectively; P < 0.001). In weight-recovered subjects with AN, osteocalcin increased (from 8.0 ± 3.05 to 11.2 ± 6.54 ng/mL; P < 0.05), whereas NTX remained elevated (from 69.0 ± 31.09 to 66.7 ± 45.5 nmol/mmol creatinine; NS). A decrease in NTX (from 70.7 ± 40.84 to 45.9 ± 22.72 nmol/mmol creatinine; NS) occurred only in the subgroup of subjects who regained menses with weight recovery.

Conclusions: Nutritional rehabilitation induces a powerful anabolic effect on bone. However, a fall of NTX and a shift from the dominant resorptive state, which we postulate involves full recovery, may involve a hormonal mechanism and require a return of menses. Nutritional rehabilitation appears to be critical to bone recovery and may explain the ineffectiveness of estrogen treatment alone on BMD in the cachectic state. Am J Clin Nutr 2007;86:92–9.

KEY WORDS Anorexia nervosa, bone mineral density, osteopenia, amenorrhea, bone markers

INTRODUCTION

Amenorrhea is a known risk factor for osteopenia, and it occurs with weight loss in persons with anorexia nervosa (AN). Women with AN are at high risk of fractures and severe osteoporosis at menopause. Studies of the efficacy of hormone therapy or oral contraceptives (OCPs) in increasing the bone mass of women with AN have not consistently shown positive results (1–5). The mechanism by which the osteopenia develops and reverses is thought to be nutritionally mediated, but it has not yet been well defined. The role of nutrition in the recovery of bone has been underestimated. Indeed, therapy consisting of OCPs or estrogen replacement was associated with continued fractures or bone loss (6, 7).

Some studies of indexes of bone turnover in women with AN have found an uncoupling of bone homeostasis, characterized by a decrease in osteoblastic function (bone formation) and an increase in osteoclastic function (bone resorption), although results have been inconsistent (6–12). The mechanisms by which bone homeostasis is disrupted and recovered in women with AN are poorly understood. Osteocalcin and N-telopeptide (NTX) are established biochemical markers of bone formation and resorption, respectively, and may be used as reliable measures of bone metabolism (13).

In this longitudinal study, we studied women with AN before and after their weight was normalized. We then compared them with healthy female control subjects and a previously studied reference population to better understand the changes in bone mineral density (BMD) and the mechanism of recovery (14; L Audi, personal communication, 23 November 2004).

SUBJECTS AND METHODS

Subjects

We studied 28 patients with AN and 11 control subjects. In addition, we compared our data with those from 30 reference control subjects (14; L Audi, personal communication, 23 November 2004). Patients were women with AN who were hospitalized for treatment at the Eating Disorder Research Unit at the New York State Psychiatric Institute, Columbia University Medical Center. All met DSM-IV criteria for AN from the 4th edition

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2 Supported by NIH, National Institute on Child Health and Human Development grants R01 HD36444-01-5 and K23 DK-02749.

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of the *Diagnostic and Statistical Manual of Mental Disorders*, except for amenorrhea in one subject who maintained regular cycles despite low weight. Patients had a mean (±SD) length of illness of 98.0 ± 59.4 mo. Subjects were recruited from referrals by physicians and mental health workers or by direct contact with the clinic. The patients with AN were 18–35 y old at hospital admission (x ± SD age: 23.0 ± 3.98 y). All patients were screened before entering the study. Patients with primary amenorrhea, polycystic ovarian syndrome, or prolactin-secreting tumors; those who were taking medications known to affect bone metabolism or reproductive function and hypothalamic-pituitary-ovarian axis or hypothalamic-pituitary-adrenal axis, including estrogen or OCPs; or those who were pregnant were excluded by physical examination and hormonal profiles. Formal exercise was not permitted during hospitalization, but no effort was made to control for previous exercise load.

The 11 healthy control subjects were recruited from the New York City area and the Columbia University campus by public advertisements. All were healthy, did not have significant medical or psychiatric histories, and were matched with patients according to age and percentage of ideal body weight (IBW) range after recovery (90–100% IBW). Their ages ranged from 18 to 36 y (x ± SD age: 24.7 ± 5.10 y). None of the control subjects had a history of an eating disorder. Potential control subjects with a history of psychiatric or medical illness or who were receiving hormonal or other medications known to affect reproductive function or bone metabolism were excluded. All of the control subjects exercised <3 h/wk and had regular menstrual cycles.

We also compared our data with reference control subjects from a study done in 2002 (14; L Audi, personal communication, 23 November 2004). That study examined 30 healthy, postpubertal white girls (x ± SD age: 18.2 ± 2.5 y) who were of Spanish descent. We used that reference group as a comparison for our subjects, because the body mass index (BMI; in kg/m²) and assays used to assess bone markers were similar.

All subjects gave written informed consent. The study protocol was approved by the Institutional Review Boards of the New York State Psychiatric Institute, Columbia University, and St Luke’s–Roosevelt Hospital Center, and procedures were followed according to approved ethical guidelines.

**Medical and menstrual histories and hormonal evaluation**

Patients were evaluated at the initiation of hospitalization for history of eating disorder and previous treatment (age at onset of AN, prior treatment type and duration, lowest and highest adult BMI, and menstrual history), current symptoms of eating disorder, general activity, and nutritional profile. A full medical history was taken, and a physical examination was performed by a physician of the Eating Disorders Unit staff. Venous blood and urine samples were taken for hormone profile analysis. All patients with secondary amenorrhea fit the criteria for hypothalamic amenorrhea: normal concentrations of prolactin, testosterone, and dehydroepiandrosterone sulfate and low-to-normal concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Patients were interviewed monthly to assess menstrual status.

Each healthy control subject completed a medical questionnaire designed to assess general medical and menstrual history and was given a brief physical examination by a study physician. Healthy control subjects were also given a take-home ovulation test kit to confirm ovulatory cycles and were asked to keep records of menstrual periods. All studies were done during the follicular phase of the menstrual cycle (days 3–10) in menstruating subjects.

**Treatment**

All patients underwent an inpatient, behavioral weight-gain treatment at the New York State Psychiatric Institute with cognitive, supportive, family, nutritional, and psychoeducational group elements aimed at restoring weight to a minimum of 90% IBW according to the 1959 Metropolitan Life Insurance Tables (15). Mean BMI at recovery was 20.5 ± 1.13. On admission, patients were fed a standard hospital diet of 1800 kcal (=55% of energy from carbohydrates, 15% of energy from protein, and 30% of energy from fat), prescribed as 3 meals/d plus a snack. Patients were expected to eat 100% of the food prescribed and were observed during and for 1 h after each meal. If they were unable to gain weight, calories were increased in 400-kcal increments in food or liquid nutritional supplement (Ensure Plus; Abbott Laboratories, Abbott Park, IL). After a brief medical stabilization period (1–2 wk), patients began the active weight-gain phase of treatment, which continued until the patients reached 90% IBW. The minimum expected rate of weight gain was 1 kg/wk, although, on average, patients tended to gain 1.6 kg/wk. At the peak of weight gain, caloric intake was ≈3700 kcal, with 3000 kcal as food and the remainder as supplement.

The weight-gain phase of treatment was followed by a 4–6-wk period of weight maintenance with increasing independence and transition to outpatient care. Mean caloric intake on discharge was 2600 kcal. For the patients in this study, the duration of inpatient treatment ranged from 26 to 112 d (x ± SD treatment duration: 65.7 ± 20.7 d). No vitamin D or calcium supplements were given.

**Biochemical analyses**

Serum osteocalcin was measured with the use of a human immunoradiometric assay (Immunotopics International, San Clemente, CA) with a sensitivity of 0.5 ng/mL and an interassay CV of 5.5–6.7%. Urine NTXs were measured with the use of an enzyme-linked immunoassay (Ostex International Inc, Seattle, WA) with a detection limit of 20 nmol bone collagen equivalent and an interassay CV of 4.1%. Blood and urine were collected twice from patients for measurements of bone markers and hormones, once at admission and once after maintenance of 90% IBW for ≥2 wk. Bone markers were measured once in control subjects.

Estradiol, FSH, and LH were measured in serum by using a solid-phase chemiluminescence immunoassay (Immulite; Diagnostic Products Co, Los Angeles, CA). Assay sensitivity was 20 pg/mL for estradiol, 0.1 mIU/mL for FSH, and 0.1 mIU/mL for LH. The intraassay and interassay CVs for estradiol were 9.3% and 10.5%, respectively. The intraassay and interassay CVs for FSH were 1.9% and 5.0%, respectively. The intraassay and interassay CVs for LH were 3.6% and 5.0%, respectively.

**Bone density and body composition**

Total-body dual-energy X-ray absorptiometry (DXA) was used to measure bone mass and bone density. The reports from the DPX-L scanner (GE Systems, Madison, WI) were analyzed with the use of version 3.6 software and were used to determine regional BMD of the hip and spine, total body bone mineral
content, and total percentage of body fat. The CVs for BMD measurements ranged from 0.5% to 1.0% (16). When measured by DXA, percentage of body fat is independent of BMD, because this value is measured directly by recognized standard means in fat depots at sites where bone is not present (17).

**Statistical analysis**

We used multiple t tests and adjusted the probability level with the use of the Bonferroni correction to compare patients, those with amenorrhea, and those who regained menses. We used independent and dependent t tests to measure differences between groups. To examine percentage increases, we performed one-sample t tests. Multiple comparisons were made with the use of the Bonferroni method, available in SPSS software (version 12; SPSS Inc, Chicago, IL). Initially, the group of patients was analyzed as one group with the use of independent t tests; later, the menstrual status of the patients was taken into account, and one group was categorized as remaining amenorrheic and the other categorized as regaining menses. For comparisons with the reference control subjects, 2-factor repeated-measures analysis of variance with Bonferroni correction was conducted.

The power calculation was based on the changes in BMD found in hypothalamic amenorrhea resulting from weight loss. On the basis of earlier data from amenorrheic dancers, 5 patients for spine BMD (t difference: 0.127 ± 0.077) would be necessary for a power of 0.80 and a level of statistical significance of 0.05 (18). We do not have data on hip BMD, but data on athletic amenorrhea treated with OCPs (19) indicated that 12 subjects are necessary to show a significant difference (SD: 4.05; effect size: 3.55%) over 1 y in Ward’s triangle. These numbers of subjects are necessary for a power of 0.80 and a level of significance of 0.05.

**RESULTS**

Thirty-seven patients and 12 healthy control subjects entered the study. Twenty-eight patients and 11 healthy control subjects completed the study. Of the 28 study patients, 8 patients regained normal menstruation at 90% IBW.

Anthropometric measures, age, and data on the onset of the eating disorder in these subjects are shown in Table 1. No significant difference was observed in age between patients at 90% IBW and control subjects. However, the patients were significantly older than the reference control subjects (age at admission: 23.0 ± 3.9 y for patients; age for reference control subjects: 18.2 ± 2.5 y; P < 0.001). No significant difference was observed in weight, BMI, or lean body mass between patients at 90% IBW and normal control subjects. BMIs of patients at 90% IBW did not differ significantly from the BMIs of the reference control subjects (ie, 20.6 ± 2.1).

We divided the patients into 2 groups according to menstrual status at the time of treatment after 90% IBW testing. Anthropometric measures and descriptive data for the patients who regained menses during recovery (n = 8) and those who remained amenorrheic (n = 20) compared with healthy control subjects are shown in Table 2. No significant differences were observed in weight, BMI, lean body mass, or percentage body fat between the group with regained menses and the amenorrheic group at admission or after weight gain. Both groups had similar weight gain (regained menses group: 27.0 lb; amenorrheic group: 21.8 lb; amenorrheic group: 21.8 lb). After weight gain, neither group differed significantly from control subjects. The mean length of illness with AN before the time of admission was 98.0 ± 59.36 mo for all patients, and no significant difference was observed between the duration of illness for patients who regained menses than for those who remained amenorrheic.

An analysis of bone markers showed that patient osteocalcin concentrations at admission did not differ significantly from those of our healthy control subjects (Table 3). The osteocalcin concentrations were significantly higher in patients with AN (8.0 ± 3.05 ng/mL) than in the reference control subjects (6.2 ± 1.90 ng/mL; P < 0.05). A significant increase of 47.93 ± 73.38% (P < 0.01) was observed in serum osteocalcin concentrations in patients with AN from low weight (8.0 ± 3.05 ng/mL) to 90% IBW (11.2 ± 6.54 ng/mL; P < 0.05), an increase that surpassed the values for the reference control group (6.2 ± 1.90 ng/mL).

Urinary NTX concentrations of patients with AN (69.0 ± 31.09 nmol/mmol creatinine) were higher at admission than those of healthy control subjects (48.3 ± 14.38 nmol/mmol creatinine; P < 0.05) and reference control subject (37.0 ± 6.00 nmol/mmol creatinine; P < 0.001). After nutritional rehabilitation, there was

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**Table 1**

<table>
<thead>
<tr>
<th>Characteristics of patients and control subjects</th>
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</thead>
<tbody>
<tr>
<td>Patients with AN (n = 28)</td>
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<tr>
<td>Control subjects (n = 11)</td>
</tr>
<tr>
<td>Age at menarche (y) 13.1</td>
</tr>
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<td>Weight (kg) 42.1 ± 4.9</td>
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<tr>
<td>BM1 (kg/m²) 16.1 ± 1.7</td>
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<td>Lean body mass (g) 35675 ± 3505</td>
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<td>Percentage body fat (%) 8.1 ± 5.5</td>
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<tr>
<td>Age at menarche (y) 15.0 ± 3.5</td>
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<tr>
<td>Age at onset of eating disorder (y) 15.0 ± 3.5</td>
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<tr>
<td>Duration of treatment (d) 65.7 ± 20.7</td>
</tr>
<tr>
<td>Duration of illness (mo) 98.0 ± 59.4</td>
</tr>
<tr>
<td>1 All values are x ± SD. Controls were at 90-100% IBW. AN, anorexia nervosa; IBW, ideal body weight; NA, not applicable.</td>
</tr>
<tr>
<td>2 Significant difference between patients with AN at admission and at 90% IBW, P &lt; 0.05 (dependent t test).</td>
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<tr>
<td>3 Significant difference between patients with AN at admission and at 90% IBW, P &lt; 0.001 (dependent t test).</td>
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<tr>
<td>4 Significant difference between patients with AN at admission and control subjects, P &lt; 0.001 (independent t test).</td>
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</table>
a decrease in NTX concentrations so that the NTX concentrations of patients with AN who reached 90% IBW (66.7 ± 45.48 nmol/mmol creatinine) did not differ from those of control subjects, although they remained higher than those of reference control subjects (37.0 ± 6.00 nmol/mmol creatinine, P < 0.001).

Bone density analysis showed that, on treatment, patients gained a significant 4.38 ± 7.48% (P < 0.05) increase in spine BMD and a 3.77 ± 8.8% (P < 0.05) increase in hip BMD after weight gain from low weight to 90% IBW in just 2.2 mo. Despite the large increases, the bone density of the patients with AN did not reach the values of control subjects and was still significantly different at 90% IBW (Table 3).

Patients on admission had significantly lower estradiol (24.4 ± 7.11 pg/mL compared with 36.0 ± 33.92 pg/mL; P < 0.05) and FSH (1.8 ± 2.15 mIU/mL compared with 3.7 ± 1.34 mIU/mL; P < 0.05) concentrations than did control subjects (Table 3), and those concentrations remained lower despite weight rehabilitation. When we examined the change in hormone concentrations only, estradiol concentrations increased significantly in patients with AN from admission (24.4 ± 7.11

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**TABLE 2**

Characteristics of groups of patients by menstrual status

<table>
<thead>
<tr>
<th></th>
<th>Regained menses at 90% IBW (n = 8)</th>
<th>Amenorrheic at 90% IBW (n = 20)</th>
<th>Control subjects (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>23.2 ± 5.0</td>
<td>23.0 ± 3.5</td>
<td>24.7 ± 5.1</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>42.4 ± 3.7</td>
<td>41.8 ± 5.4</td>
<td>56.5 ± 5.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>16.6 ± 2.1</td>
<td>16.0 ± 1.5</td>
<td>21.5 ± 0.9</td>
</tr>
<tr>
<td>Lean body mass (g)</td>
<td>34898 ± 3154</td>
<td>35987 ± 3666</td>
<td>38401 ± 3776</td>
</tr>
<tr>
<td>Percentage body fat (%)</td>
<td>11.1 ± 7.1</td>
<td>7.0 ± 4.3</td>
<td>27.8 ± 4.6</td>
</tr>
<tr>
<td>Age at menarche (y)</td>
<td>12.8 ± 4.2</td>
<td>13.3 ± 1.6</td>
<td>12.9 ± 1.0</td>
</tr>
<tr>
<td>Duration of illness (mo)</td>
<td>114.0 ± 56.7</td>
<td>94.1 ± 61.9</td>
<td>NA</td>
</tr>
<tr>
<td>Duration of treatment (d)</td>
<td>73.4 ± 20.8</td>
<td>62.6 ± 16.8</td>
<td>NA</td>
</tr>
</tbody>
</table>

All values are x ± SD. Control subjects were at 90-100% IBW. AN, anorexia nervosa; IBW, ideal body weight; NA, not applicable. Analysis involved multiple t tests, and probability levels were adjusted by using the Bonferroni correction.

---

**TABLE 3**

Bone marker, bone density, and hormone data

<table>
<thead>
<tr>
<th></th>
<th>Patients with AN (n = 28)</th>
<th>Control subjects (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Admission</td>
<td>90% IBW</td>
</tr>
<tr>
<td>Osteocalcin (ng/mL)</td>
<td>8.0 ± 3.05</td>
<td>11.2 ± 6.54</td>
</tr>
<tr>
<td>Urine NTX (nmol/mmol Cr)</td>
<td>69.0 ± 31.09</td>
<td>66.7 ± 45.48</td>
</tr>
<tr>
<td>BMD spine (g/cm²)</td>
<td>0.914 ± 0.147</td>
<td>0.954 ± 0.136</td>
</tr>
<tr>
<td>BMD hip (g/cm²)</td>
<td>0.928 ± 0.125</td>
<td>0.963 ± 0.114</td>
</tr>
<tr>
<td>Total BMD (g/cm²)</td>
<td>1.060 ± 0.086</td>
<td>1.061 ± 0.081</td>
</tr>
<tr>
<td>LH (mIU/mL)</td>
<td>1.5 ± 3.18</td>
<td>2.7 ± 2.95</td>
</tr>
<tr>
<td>FSH (mIU/mL)</td>
<td>1.8 ± 2.15</td>
<td>2.5 ± 1.25</td>
</tr>
<tr>
<td>Testosterone (ng/dL)</td>
<td>76.0 ± 37.43</td>
<td>79.1 ± 35.24</td>
</tr>
<tr>
<td>Estradiol (pg/mL)</td>
<td>24.4 ± 7.11</td>
<td>35.0 ± 14.45</td>
</tr>
<tr>
<td>Prolactin (ng/mL)</td>
<td>8.6 ± 3.54</td>
<td>9.3 ± 6.91</td>
</tr>
<tr>
<td>DHEAS (µg/dL)</td>
<td>—</td>
<td>143.6 ± 84.50</td>
</tr>
</tbody>
</table>

All values are x ± SD; percentage of change in parentheses. Control subjects were at 90-100% IBW. AN, anorexia nervosa; IBW, ideal body weight; NTX, N-telopeptide; BMD, bone mineral density; LH, luteinizing hormone; FSH, follicle-stimulating hormone; DHEAS, dehydroepiandrostosterone sulfate.

---

1 Significant difference between patients with AN at admission and at 90% IBW, P < 0.05 (dependent t test).
2 Significant difference between patients with AN at admission and at 90% IBW, P < 0.01 (dependent t test).
3 Significant difference between patients with AN at admission and control subjects, P < 0.05 (independent t test).
4 Significant difference between patients with AN at admission and control subjects, P < 0.05 (independent t test).
5 Significant difference between patients with AN at admission and control subjects, P < 0.01 (independent t test).
6 Significant difference between patients with AN at admission and control subjects, P < 0.05 (independent t test).
7 Significant difference between patients with AN at admission and control subjects, P < 0.01 (independent t test).
8 Significant difference between patients with AN at admission and control subjects, P < 0.05 (independent t test).
Estradiol (pg/mL) to 90% IBW
Testosterone (ng/dL) 86.8
LH (mIU/mL) 50512
BMD hip (g/cm²) to 90% IBW (35.0
Urine NTX (nmol/mmol Cr) 50512
BMD spine (g/cm²) 50512
Osteocalcin (ng/nL)

TABLE 4
Bone marker, bone density, and hormonal data by menstrual status

<table>
<thead>
<tr>
<th></th>
<th>Regained menses at 90% IBW (n = 8)</th>
<th>Amenorrheic at 90% IBW (n = 20)</th>
<th>Control subjects (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Admission 90% IBW</td>
<td>Admission 90% IBW</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ostecalcin (ng/mL)²⁻⁷</td>
<td>5.9 ± 1.49</td>
<td>9.5 ± 3.84 (69.8 ± 89.55)</td>
<td></td>
</tr>
<tr>
<td>Urine NTX (nmol/mmol Cr)³⁻⁷</td>
<td>70.7 ± 40.84</td>
<td>64.6 ± 22.72 (–28.2 ± 27.4)</td>
<td></td>
</tr>
<tr>
<td>BMD spine (g/cm²)⁶⁻¹⁰</td>
<td>0.959 ± 0.141</td>
<td>1.003 ± 0.132 (4.59 ± 6.38)</td>
<td></td>
</tr>
<tr>
<td>BMD hip (g/cm²)⁶⁻¹⁰</td>
<td>0.972 ± 0.085</td>
<td>1.002 ± 0.083 (3.08 ± 2.35)</td>
<td></td>
</tr>
<tr>
<td>Total BMD (g/cm²)⁷⁻¹¹</td>
<td>1.092 ± 0.027</td>
<td>1.095 ± 0.029 (0.27 ± 7.41)</td>
<td></td>
</tr>
<tr>
<td>LH (mIU/mL)²⁻¹²</td>
<td>3.9 ± 3.56</td>
<td>3.5 ± 3.25</td>
<td></td>
</tr>
<tr>
<td>FSH (mIU/mL)³⁻¹²</td>
<td>3.0 ± 2.86</td>
<td>2.4 ± 1.67</td>
<td></td>
</tr>
<tr>
<td>Testosterone (mg/dL)</td>
<td>86.8 ± 34.60</td>
<td>89.0 ± 31.82</td>
<td></td>
</tr>
<tr>
<td>Estradiol (pg/mL)⁷⁻¹³</td>
<td>29.3 ± 10.32</td>
<td>47.5 ± 15.74</td>
<td></td>
</tr>
<tr>
<td>Prolactin (ng/mL)</td>
<td>8.5 ± 3.47</td>
<td>11.2 ± 9.34</td>
<td></td>
</tr>
<tr>
<td>DHEAS (μg/dL)</td>
<td>183.6 ± 30.14</td>
<td>150.2 ± 59.27</td>
<td></td>
</tr>
</tbody>
</table>

¹ All values are ± SD; percentage of change in parentheses. Control subjects were at 90-100% IBW. AN, anorexia nervosa; IBW, ideal body weight; NTX, N-telopeptide; BMD, bone mineral density; LH, luteinizing hormone; FSH, follicle-stimulating hormone; DHEAS, dehydroepiandrosterone sulfate.
² Significant difference between the regained-menses group at admission and the amenorrheic group at admission, P < 0.05 (independent t test).
³ Percentage of change from admission to 90% IBW was calculated by using a one-sample t test with Bonferroni correction.
⁴ The percentage of change did not differ significantly between the amenorrheic and the regained-menses groups.
⁵ Significant difference between the regained-menses group at admission and at 90% IBW, P < 0.05 (dependent t test).
⁶ Significant difference in percentage of change between the regained-menses group and the amenorrheic group at 90% IBW, P < 0.05 (Mann-Whitney test).
⁷ Significant difference between the amenorrheic group at admission and at 90% IBW, P < 0.05 (dependent t test).
⁸ Significant difference between the amenorrheic group at admission and control subjects, P < 0.01 (independent t test).
⁹ Significant difference between the amenorrheic group at 90% IBW and control subjects, P < 0.05 (independent t test).
¹⁰ Significant difference between the amenorrheic group at admission and at 90% IBW, P < 0.001 (dependent t test).
¹¹ Significant difference between the amenorrheic group at admission and control subjects, P < 0.05 (independent t test).
¹² Significant difference between the amenorrheic group at admission and control subjects, P < 0.001 (independent t test).
¹³ Significant difference between the regained-menses group and the amenorrheic group at 90% IBW, P < 0.05 (independent t test).

As seen in the subanalysis of menstrual status, osteocalcin concentrations rose with weight gain in both groups (Figure 1; Table 4). The rise in osteocalcin did not differ significantly between the regained menses group and the amenorrheic group. However, the amenorrheic group continued to have higher values than did our reference control subjects (P < 0.02), which indicated a powerful effect of weight gain in this group (11.9 ± 7.33 ng/mL compared with 6.2 ± 1.90 ng/mL; P < 0.001). The higher NTX concentrations seen in subjects with persistent amenorrhea (75.0 ± 49.94 nmol/mmol creatinine) did not differ significantly from those in healthy control subjects, but they were significantly higher than the reference control population even after weight gain (37.0 ± 6.00 nmol/mmol creatinine; P < 0.02) (Figure 2). As expected, patients with return of menses at 90% IBW showed a fall in NTX (from 70.7 ± 40.84 to 45.9 ± 22.72 nmol/mmol Cr; NS) that reached into the ranges in healthy control subjects (45.9 ± 22.72 compared with 48.3 ± 14.38 nmol/mmol Cr; P < 0.09) (Table 4) and the reference control subjects (45.9 ± 22.72 compared with 37.0 ± 6.00 nmol/mmol Cr; P = 1.00) (Figure 2). None of these changes were statistically significant because of the large SD and the small number of subjects. No significant time-by-group interaction was observed for either group. However, a significant percentage fall in NTX was observed in the group that regained menses (–28.2 ± 27.4%; P < 0.05) (Table 4).
In the menstrual status subanalysis (Table 4), LH and FSH hormones differed significantly ($P < 0.001$) at baseline, with lower concentrations in the amenorrheic group than in the healthy control subjects. In the amenorrheic group, a nonsignificant rise in LH and FSH concentrations was observed.

With respect to menstrual status (Table 4), the patients who regained menses did show an increase of estradiol into the normal range and did not significantly differ from the healthy control subjects ($47.5 \pm 15.74$ compared with $56.0 \pm 33.92$ pg/mL; NS) (Table 4). Those who remained amenorrheic had a significant rise in estradiol between admission ($22.5 \pm 4.49$ pg/mL) and the achievement of 90% IBW ($30.0 \pm 10.63$ pg/mL; $P < 0.05$) (Table 4), but, at 90% IBW, their estradiol concentrations remained significantly lower than those in the recovered-menses group ($30.0 \pm 10.63$ pg/mL and $47.5 \pm 15.74$ pg/mL, respectively). This suggests, along with the lower gonadotrophs at admission for treatment of anorexia nervosa until recovery of 90% of ideal body weight (IBW) in subjects who regained menses and those who remained amenorrheic. Bold lines represent $\pm 2$ SDs from the mean NTX values of $37.00 \pm 6.00$ nmol/mmol Cr in reference control subjects (14; L Audi, personal communication, 23 November 2004). The error bars represent $\pm 2$ SDs. Two-factor repeated-measures ANOVAs with Bonferroni corrections were used for comparisons to the reference control subjects. Time-by-group interaction was not significant; values in the amenorrheic group were compared with those in the reference control group ($P < 0.03$).

Mean age was $18.2 \pm 2.5$ y, and mean BMI (in kg/m$^2$) was $20.6 \pm 2.1$.

![Figure 2](image.png)

**FIGURE 2.** Mean ($\pm$SD) changes in urine N-telopeptide (NTX) concentrations from admission for treatment of anorexia nervosa until recovery of 90% of ideal body weight (IBW) in subjects who regained menses and those who remained amenorrheic. Bold lines represent $\pm 2$ SDs from the mean NTX values of $37.00 \pm 6.00$ nmol/mmol Cr in reference control subjects (14; L Audi, personal communication, 23 November 2004). The error bars represent $\pm 2$ SDs. Two-factor repeated-measures ANOVAs with Bonferroni corrections were used for comparisons to the reference control subjects.

In the menstrual status subanalysis (Table 4), LH and FSH concentrations from admission for treatment of anorexia nervosa until recovery of 90% of ideal body weight (IBW) in subjects who regained menses and those who remained amenorrheic. Bold lines represent $\pm 2$ SDs from the mean NTX values of $37.00 \pm 6.00$ nmol/mmol Cr in reference control subjects (14; L Audi, personal communication, 23 November 2004). The error bars represent $\pm 2$ SDs. Two-factor repeated-measures ANOVAs with Bonferroni corrections were used for comparisons to the reference control subjects. Time-by-group interaction was not significant; values in the amenorrheic group were compared with those in the reference control group ($P < 0.03$).

Mean age was $18.2 \pm 2.5$ y, and mean BMI (in kg/m$^2$) was $20.6 \pm 2.1$.

**DISCUSSION**

This is the first longitudinal study to show significant percentage increases in BMD ($4.38 \pm 7.48\%$ for spine; $3.77 \pm 8.8\%$ for hip; $0.09 \pm 5.81\%$ for total; $P < 0.05$ for all) with nutritional therapy over a period of 2.2 mo in women with AN, which suggests a powerful anabolic effect of nutritional therapy. This is also the first longitudinal study to show that mildly depressed osteocalcin concentrations increase with weight gain, whereas elevated NTX concentrations fall into the normal range only with resumption of menses. Although the osteocalcin concentrations are not significantly depressed, they do not increase parallel to the increase in resorption (NTX concentrations) until nutritional rehabilitation is achieved. These findings suggest that the recovery of bone metabolism is biphasic, involving a primary nutritional mechanism that stimulates bone formation and a hormonal mechanism that decreases bone resorption.

Although no differences in BMD improvement were observed between the patients who resumed menses and those who did not, and although the 2 groups gained weight to a similar level, we postulate that, in the former group, larger increases in BMD would be seen with a longer period of observation because of normalization of NTX and thus bone resorption. These studies were done with regional BMD measurements; site-specific measurements could provide more accurate data.

Our findings may also explain the lack of effect of estrogen (3) and OCP treatments (4) on bone metabolism in the cachectic state, because bone metabolism may not recover fully until a normal nutritional state has been established. However, larger studies are needed to confirm and extend these observations, because our control group was small.

Most studies support the notion that the mechanism of osteopenia in AN is nutritionally related (9, 20, 21). Our data are consistent with studies of healthy women that show a depression of bone formation indexes with as little as 5 d of nutritional deprivation. Rapid increases in indexes of bone resorption are also seen with more severe nutritional restriction (22). The changes in markers of bone resorption exactly parallel our findings (22). Conversely, nutritional rehabilitation of women with AN confirms the fact that recovery of bone formation occurs first, and that it is followed by suppression of resorption (10, 23). This chronology suggests that therapy with antiresorptives such as estrogen may not be effective without appropriate nutritional therapy. It also may explain the continued fractures in women taking OCPs that are reported in the literature (6, 7) and the lack of effect of estrogen on bone density in AN (3). Antiresorptive therapy may, however, have a therapeutic role after weight gain but before return of menses when bone formation has resumed.

Our data are also consistent with other studies indicating that, despite recovery from AN, osteopenia persists (6, 10, 14, 24). In the present study, however, we were surprised by increases in spine and hip bone densities of 3–4.4% over 2.2 mo, a greater and more rapid response than was seen in previous studies (10, 25). The current study used a regional analysis of total BMD, in which the defined regions of interest are less precise than are dedicated hip and spine BMD measurements. However, these methods have been used with good precision to evaluate regional BMD in athletes (26, 27). Because our studies used the same machines and operators in a longitudinal fashion, we consider the results to be highly significant.

Weight gain and changes in body composition may affect the accuracy of DXA scans, although, in a detailed study of this problem that used measurements made by a Lunar machine, the changes were not clinically significant (28). Because of a spurious increase in bone area, these studies show an increase in bone mineral content with weight gain, whereas BMD decreases. Thus, our findings may be slightly attenuated by weight gain, although there is no method for compensating for this.

To further understand the mechanism of persistent osteopenia in AN, hormones and markers of bone turnover were examined. Our results indicate that, at low weight, patients with AN have normal-to-increased bone formation, as indicated by osteocalcin concentrations, which appears to be insufficient to match the amount of bone resorption, as evidenced by increased NTX. This results in an imbalance in bone turnover and leads to osteopenia. These data are similar to findings from previous studies (6, 11, 12, 14, 20, 21). Grinspoon et al (12) compared patients with AN with patients with hypothalamic amenorrhea and healthy control subjects in a cross-sectional study. Both patients with AN and patients with hypothalamic amenorrhea had higher bone resorption indexes than did control subjects, but the indexes in patients with AN were significantly higher. Unlike the present study, that

**REFERENCES**

study showed lower serum osteocalcin concentrations in patients with AN than in healthy control subjects.

It is interesting that rapid increases in bone density were seen in both groups of anorexic patients—those who regained menses and those who remained amenorrheic. Our hormone data suggest that the group that remained amenorrheic had greater suppression of ovarian function, even at baseline, than did the group that regained menses, as evidenced by significantly lower LH concentrations at baseline and a lack of estradiol rise into the normal range. LH suppression would be expected to compromise ovarian stimulation and, in turn, estradiol secretion. Because estradiol decreases bone resorption, its suppression would favor an increase in NTX, which is consistent with our data. Followed over a longer period of time, the subjects who remained amenorrheic would likely have a greater degree of osteopenia than would those who regained menses.

The difference manifested at baseline between the 2 groups may explain the lack of significance of the time-by-group interaction, because the groups were defined by outcome and not by treatment. There may be underlying physiologic differences at baseline that affect their response and that have not yet been determined because this was not a controlled intervention.

Some studies have implicated insulin-like growth factor 1, a nutritionally dependent bone trophic factor, as the major factor contributing to osteopenia in AN (9, 10). One study showed a modest increase in spine bone density in patients treated with recombinant insulin-like growth factor 1 and OCPs compared with that in patients taking placebo plus OCPs (4). It is interesting that bone density increased 1.8% after 9 mo of therapy (4), a significantly lower increase than we saw in our patients after 2.2 mo of nutritional therapy. With reversal of amenorrhea, studies have shown a 6–25% increase in bone density (6, 7, 23, 29). This is in contrast to studies that have shown a 4.1–4.9% increase in BMD with bisphosphonates (30).

Given the high prevalence of osteopenia in women with AN, the major clinical concern is the progression to osteoporosis and pathologic fractures (31). One study estimated a fracture rate of 7 times normal in women and men with AN (25). A long-term cohort study that followed 208 women with AN for a mean of 12.9 y showed a 57% cumulative risk of fracture at 40 y after diagnosis (32). Fractures are a serious complication from osteopenia in AN and are associated with significant morbidity.

AN affects young women at a critical time for the development of peak bone mass, and it is often a chronic disease with a high relapse rate. As a result, the complication of osteopenia is often profound. A better understanding of the pathogenesis of osteopenia in AN will allow the development of prophylactic or therapeutic treatments to prevent pathologic fractures. Our data suggest that nutritional therapy is critical and necessary for optimal effect of other therapies such as estrogen replacement or other antiresorptives. Nutritional therapy also exerts an anabolic effect, which appears to be more powerful than the use of insulin-like growth factor 1 with OCPs. Treatment with antiresorptives, including estrogen replacement and OCPs, should be reexamined, because these agents do not improve bone formation and may not be effective unless bone formation is stimulated. Our observations may be important to an understanding of the mechanism of possible reversal of osteoporosis in AN, for which there is as yet no effective treatment.

We thank Laura Audi (Hospital Vall d’Hebron Unidad Investigacion Endocrinologia Pediatrica, Barcelona, Spain) for her generosity in allowing the use of her normal data for comparison with our subjects.

The authors’ responsibilities were as follows—JD: collected data and wrote the manuscript; LG: collected and analyzed data; SSG: researched the literature and collected data; LM: conducted clinical trial on patients with anorexia; SFE: assisted in conducting clinical trial, including refeeding of subjects with anorexia; BTW: supervised the treatment of subjects with anorexia nervosa; JW: conducted bone mineral density measurements; RP: conducted bone mineral density and body composition measurements; MPW (primary investigator): organized and conducted the clinical trial, organized and reviewed the data analyses and the literature research, and wrote the manuscript. None of the authors had a personal or financial conflict of interest.

REFERENCES


Independent associations of insulin resistance with high whole-body intermuscular and low leg subcutaneous adipose tissue distribution in obese HIV-infected women¹⁻³

Jeanine B Albu, Sonjia Kenya, Qing He, Marsha Wainwright, Evan S Berk, Stanley Heshka, Donald P Kotler, and Ellen S Engelson

ABSTRACT

Background: Obesity and insulin resistance are growing problems in HIV-positive (HIV+) women receiving highly active antiretroviral therapy (HAART).

Objective: The objective was to determine the contribution of adipose tissue (AT) enlargement and distribution to the presence of insulin resistance in obese HIV+ women.

Design: Whole-body intermuscular AT (IMAT), visceral AT (VAT), subcutaneous AT (SAT), and SAT distribution (leg versus upper body) were measured by whole-body magnetic resonance imaging. Insulin sensitivity ($S_I$) was measured with an intravenous glucose tolerance test in obese HIV+ women recruited because of their desire to lose weight ($n = 17$) and in obese healthy controls ($n = 32$).

Results: The HIV+ women had relatively less whole-body SAT and more VAT and IMAT than did the controls ($P < 0.05$ for all). A significant interaction by HIV status was observed for the relation of total SAT with $S_I$ ($P < 0.001$ for the regression’s slope interactions after adjustment for age, height, and weight). However, relations of IMAT, VAT, and SAT distribution (leg SAT as a percentage of total SAT; leg SAT%) with $S_I$ did not differ significantly between groups. For both groups combined, the best model predicting a low $S_I$ included significant contributions by both high IMAT and low leg SAT%, independent of age, height, and weight, and no interaction between groups was observed (overall $r^2 = 0.44, P = 0.0003$).

Conclusion: In obese HIV+ women, high whole-body IMAT and low leg SAT% distribution are independently associated with insulin resistance.

KEY WORDS Subcutaneous adipose tissue, intermuscular adipose tissue, adipose tissue distribution, insulin resistance, HIV infection

INTRODUCTION

Insulin resistance is a central feature of the metabolic syndrome (1, 2). Generalized and regional lack of body fat in congenital lipodystrophies (3) and upper-body fat accumulation in obesity (4) have been shown to be associated with insulin resistance in HIV-positive (HIV+) individuals. A syndrome of fat redistribution consisting of peripheral fat loss (face, limbs, and buttocks) and central fat accumulation (abdomen, breast, and dorsocervical area) has been described in HIV+ men and women (5, 6). HIV+ men and women with fat redistribution were more insulin resistant than were those without such redistribution (7–19). Obesity has emerged as a growing problem in HIV+ individuals receiving highly active antiretroviral therapy (HAART) (20), particularly in women (21, 22). The influence of the enlargement and distribution of various adipose tissue (AT) compartments on the presence of insulin resistance has not been clearly established in obese HIV+ women. However, its influence could be important for assessing the response to interventions that alter body fat, which may be useful in HIV-negative (HIV−) but not in obese HIV+ women (5, 23–25).

Most studies reporting on the relation between fat distribution and insulin resistance have compared groups of HIV+ individuals with or without lipodystrophy as determined a priori by observational criteria (7, 8, 11–13, 17–19). Studies using continuous variables (9–11, 14–16) found correlations between lipodystrophy measured by dual-energy X-ray absorptiometry (DXA), ie, more trunk fat or less leg fat as a percentage of total body fat and insulin resistance in both HIV+ men and women (9, 11, 15–19). Whether these associations were similar in HIV+ and HIV− cohorts has not been noted (15). DXA did not separate fat in subcutaneous AT (SAT) from fat in the visceral AT (VAT) compartment or inside muscle and organs (26–28). Separation of AT depots at the waist level by computed tomography (CT) or magnetic resonance imaging (MRI) provided additional information (26, 27); however, the findings of both positive and negative associations of SAT and VAT areas at the waist level with insulin resistance (14, 16, 26, 27, 29, 30) may have been due to differences in sex or degree of overweight (29, 31). In previous studies in patients with HIV lipodystrophy, in which whole-body AT depots were measured by whole-body MRI, relations to corresponding insulin resistance indexes were not reported (32–35). In particular, a relation of insulin resistance to SAT distribution (ie, SAT of legs versus upper body) and to IMAT [ie, subfascial, ²]}
intermuscular AT (IMAT) measured by whole-body MRI has not been reported previously in HIV+ women (32–35). Higher IMAT was found to be independently associated with insulin resistance in healthy premenopausal women (28), and decreases in leg fat or thigh SAT were shown to be independently associated with unfavorable glucose and lipid concentrations in larger cohorts not characterized by HIV status (36, 37).

Therefore, the overall aim of this study was to determine the relations of total SAT, VAT, IMAT, and SAT distribution (legs versus upper body) measured by whole-body MRI in obese HIV+ women with insulin resistance. Specifically, we asked whether IMAT and SAT distribution were independently associated with insulin resistance in obese HIV+ women. We also determined whether such relations were similar to those found in healthy controls.

SUBJECTS AND METHODS

HIV+ subjects

The HIV+ women were recruited for a study of weight loss and exercise. The data presented here are the baseline data from a subset of the recruited subjects who were not diabetic and who had adequate venous access for testing (n = 17). The body mass index (BMI; in kg/m²) of the women was 30–38. Three of the women were non-Hispanic white, 3 were Hispanic, and 11 were African American. The HIV+ women were obese (BMI > 30) and clinically stable. Most of the subjects were on an antiretroviral drug regimen for ≥4 wk before enrollment and had no plans to change the regimen during the study period. The women were allowed to take their HIV medications as prescribed. Of the 17 HIV+ women whose data are presented here, 4 women were not receiving HAART, 13 were taking nucleoside reverse transcriptase inhibitors, 9 were taking protease inhibitors (PIs), and 9 were taking non-nucleoside reverse transcriptase inhibitors. Exclusion criteria were as follows: 1) any active opportunistic infection or malignancy, 2) pregnancy or breastfeeding, 3) uncontrolled hypertension, 4) history of MRI or any condition that would prevent exercise, 5) diabetes mellitus, and 6) a self-reported medical history of an eating disorder (eg, anorexia nervosa or bulimia nervosa), gallbladder disease, renal disease, active substance abuse, or methadone treatment. The women chosen for the present analysis were either pre- or perimenopausal and none of them were receiving estrogen replacement therapy. All subjects had an intravenous glucose tolerance test (IVGTT) performed within 10 d of the beginning of a regular menstrual period. The women were also weight stable for ≥6 wk before the measurements.

Healthy controls

The women in the control group 1 (n = 12) and in control group 2 (n = 20) were recruited for 2 other studies. Data from measurements that were identical to those performed for the HIV+ women (except for the insulin sensitivity measurements in control group 2; see below) were used in the analyses. For both control groups, everyone with a BMI ≥ 30 (obese, as in the HIV+ cohort) and for whom data were available was included in the study. In group 1, 5 of the women were non-Hispanic white, 1 was Hispanic, and 6 were African American. In group 2, 7 of the women were non-Hispanic white and 13 were African American. The women in both control groups were weight stable for 6 mo before the studies, were healthy, were premenopausal, were non-diabetic, had regular menstrual cycles, reported no medical problems, reported no symptoms, were taking no medications, and had normal results from blood count and chemistry panels. No HIV test was performed in these groups. All subjects signed an informed consent form; the protocol and consent form were approved by the St Luke’s–Roosevelt Hospital Institutional Review Board and Radiation Safety Committee.

Anthropometric measures

Body weight was measured to the nearest 0.1 kg (Avery Weigh-Tronix, Fairmont, MN) and height to the nearest 0.5 cm with a stadiometer (Holtain, Crosswell, United Kingdom).

Whole-body magnetic resonance imaging

AT compartments and skeletal muscle (SM) volumes were measured on a 1.5T MRI scanner (6X Horizon; General Electric, Milwaukee, WI) as described previously (38–40). The entire body was visualized on a scout coronal image (6X Horizon), and the axial level of L₄–L₅ was identified. The scans were acquired by using contiguous axial slices of 10-mm thickness at 40-mm intervals below L₄–L₅ to the toes, and above this level to the fingertips (=40–50 images for women of average height). Images were then analyzed on a PC platform as described (28, 32–35, 38–40). Briefly, the procedure for calculating AT volume is to first measure the relevant tissue area in each slice with the use of threshold methods and manual delineation to draw boundaries among different tissues. The volume between slices is extrapolated from the area measurements. The following volumes were calculated: VAT, total SAT, and IMAT. We defined IMAT as the AT visible between the muscle groups and beneath the muscle fascia (38). The gray level intensity (threshold value) of the AT in the SAT region was first determined and used as reference. This threshold value was reduced by 20% to identify IMAT threshold.

In addition to whole-body results, regional values for subcutaneous AT were determined for the arms, legs, and upper and lower trunk. The regional subcutaneous AT volumes used in this article delineated SAT volume in the legs (leg SAT, ie, all SAT inferior to the greater trochanter, including the greater trochanter area) and SAT volume in the upper body (upper SAT, ie, all SAT superior to the level of the trochanter, including the arms). The MRI scans were read at the New York Obesity Research Center Image Reading Center at St Luke’s Roosevelt Hospital Center. The CV on repeated readings of the same 2 scans by observers analyzing the images was 3.8% for SAT, 3.4% for SAT, 9.7% for VAT, 2.2% for SM, and 7.3% (estimate) for IMAT.

Insulin sensitivity index determined by intravenous glucose tolerance test

The Bergman minimal model was used to quantify Sₙ (MINMOD 2.0; copyright RN Bergman, 1986; 41). This measurement was made in all subjects during the follicular phase of the menstrual cycle. Glucose (0.3 g/kg, 50% dextrose injection; Abbott, North Chicago, IL) was administered intravenously at time 0 min. This was followed by an injection of tolbutamide (Orinase Diagnostic, Upjohn, Kalamazoo, MI) at time 20 min in control group 2. In the HIV+ group and in control group 1, 0.03 units insulin/kg (Humulin R; Lilly Inc, Indianapolis, IN) was used due to the lack of availability of tolbutamide. Blood samples, collected through a catheter placed in the contralateral arm,
occurred at fasting and at 26 time points over the 3 h after glucose administration. Plasma glucose and insulin were measured in all samples, and the $S_I$ was calculated from these values with the nonlinear mathematical model of glucose disappearance. Studies in the literature have shown that the $S_I$ measured at 20 min in the same subject with the use of IVGTT with intravenous insulin is lower than the $S_I$ measured with the use of IVGTT with intravenous tolbutamide; however, the difference between methods (14%) appears to be constant throughout the range of insulin sensitivity in nondiabetic subjects (42). Therefore, we used a 14% lower value than that determined by the computer program for the subjects who had an IVGTT with tolbutamide at 20 min (control group 2), and the data were pooled. Furthermore, we computed possible interactions with the IVGTT method using a categorical factor denoting the IVGTT method for all relevant analyses (see Data analysis below).

### Data analysis

Data were expressed as means ± SDs for t test comparisons in the 3 experimental groups and were expressed as means ± SEMs for the analysis of covariance (ANCOVA) results. Log transformations were used for variables for which deviation from normality of distribution was found. Independent $t$ tests were used to compare measurements in the HIV+ group with those of control groups 1 and 2. Because there were 3 groups being compared by $t$ test, a Bonferroni adjustment was used. Regression lines between dependent variables and covariates were tested for interactions before ANCOVA was performed. A general linear model was used to test interactions between slopes of regression for continuous variables in the HIV+ group compared with the control groups. Although use of a mathematical correction to compute $S_I$ in control group 2 does not ensure that values would be comparable between groups, the literature suggests that rank-order correlation comparisons are preserved independently of the IVGTT method (42). In addition, as described above, a categorical factor denoting the IVGTT method (HIV+, HIV− group 1, and HIV− group 2) was entered in the ANCOVA and general linear model analyses of $S_I$, and possible interactions were computed and reported if present. Analyses were done by using STATISTICA 6.0 (Statsoft Inc, Tulsa, OK). $P < 0.05$ was considered to be statistically significant.

### RESULTS

Subject characteristics and whole-body MRI and metabolic measurements are shown in Table 1. The HIV+ women were less heavy and had less SAT than did the women in control group 1 but had significantly more VAT than did the women in both control groups (Table 1). Fasting glucose and insulin and insulin sensitivity values in the HIV+ group did not differ significantly from those of either control group.

The relative AT distribution (relative accumulation of AT in SAT, VAT, and IMAT compartments) in HIV+ women compared with that in the women in the 2 control groups combined is shown in Table 2. In contrast with Table 1, the data in the control groups were combined because measurement methods and group variances were homogenous. Data for individual AT compartment sizes (SAT, VAT, and IMAT) were adjusted for degree of overweight and for total AT. SAT was lower and both VAT and IMAT were higher in the HIV+ group than in the controls after adjustment for age, height, weight, and total AT ($P < 0.05$).

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>HIV+ group (n = 17)</th>
<th>Control group 1 (n = 12)</th>
<th>Control group 2 (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>39.5 ± 7.5</td>
<td>36.7 ± 6.3</td>
<td>37.2 ± 6.4</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>91.5 ± 10.1</td>
<td>106.8 ± 9.9</td>
<td>91.7 ± 10.6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>34.8 ± 3.1</td>
<td>38.6 ± 2.8</td>
<td>34.1 ± 3.4</td>
</tr>
<tr>
<td>SM (L)</td>
<td>23.3 ± 3.6</td>
<td>25.0 ± 3.7</td>
<td>24.4 ± 3.3</td>
</tr>
<tr>
<td>SAT (L)</td>
<td>41.2 ± 9.8</td>
<td>52.9 ± 8.7</td>
<td>40.1 ± 6.8</td>
</tr>
<tr>
<td>Upper SAT (L)</td>
<td>24.3 ± 4.7</td>
<td>32.1 ± 5.0</td>
<td>23.4 ± 4.9</td>
</tr>
<tr>
<td>Leg SAT (L)</td>
<td>16.9 ± 5.6</td>
<td>20.8 ± 4.8</td>
<td>16.7 ± 3.0</td>
</tr>
<tr>
<td>VAT (L)</td>
<td>3.7 ± 1.2</td>
<td>2.4 ± 0.9</td>
<td>2.7 ± 1.0</td>
</tr>
<tr>
<td>IMAT (L)</td>
<td>2.3 ± 0.7</td>
<td>2.2 ± 0.5</td>
<td>1.9 ± 0.7</td>
</tr>
<tr>
<td>Fasting glucose (pmol/L)</td>
<td>5.4 ± 0.6</td>
<td>5.3 ± 0.4</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>Fasting insulin (pmol/L)</td>
<td>132 ± 107</td>
<td>84 ± 21</td>
<td>86 ± 32</td>
</tr>
<tr>
<td>$S_I$ (µU·mL⁻¹·min⁻¹)</td>
<td>1.6 ± 1.2</td>
<td>1.3 ± 0.9</td>
<td>2.2 ± 1.2</td>
</tr>
</tbody>
</table>

All values are $\bar{x} ± SD$. SM, skeletal muscle; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; IMAT, intramuscular adipose tissue; $S_I$, insulin sensitivity index.

### Table 2

<table>
<thead>
<tr>
<th>Adipose tissue distribution in HIV-positive (HIV+) and control women$^*$</th>
<th>HIV+ women (n = 17)</th>
<th>Control women (n = 32)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM (L)$^2$</td>
<td>23.9 ± 0.7</td>
<td>24.4 ± 0.5</td>
<td>0.566</td>
</tr>
<tr>
<td>SAT (L)$^3$</td>
<td>42.8 ± 0.3</td>
<td>44.1 ± 0.2</td>
<td>0.003</td>
</tr>
<tr>
<td>Leg SAT% (of total AT)$^4$</td>
<td>35.0 ± 1.3</td>
<td>37.0 ± 1.0</td>
<td>0.21</td>
</tr>
<tr>
<td>Leg SAT% (of total SAT)$^4$</td>
<td>40.1 ± 1.3</td>
<td>40.8 ± 1.0</td>
<td>0.65</td>
</tr>
<tr>
<td>VAT (L)$^5$</td>
<td>3.5 ± 0.2</td>
<td>2.6 ± 0.2</td>
<td>0.007</td>
</tr>
<tr>
<td>IMAT (L)$^5$</td>
<td>2.4 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>0.043</td>
</tr>
</tbody>
</table>

All values are $\bar{x} ± SEM$. SM, skeletal muscle; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; IMAT, intramuscular adipose tissue. All SAT inferior to the level of the greater trochanter.

$^*$ All values are adjusted for age, height, and weight.

$^2$ Measured by whole-body magnetic resonance imaging.

$^3$ Values adjusted for age, height, and weight, total AT, and SM.

$^4$ Leg SAT as a percentage of total adipose tissue (SAT + VAT + IMAT) or as a percentage of total SAT. Values were unadjusted.
weight and $r = -0.24 (P = 0.18)$ for total AT. In the HIV+ group, correlations were $r = 0.47 (P = 0.054)$ for body weight and $r = 0.64 (P = 0.006)$ for total AT, ie, lower values for leg SAT% were seen at lower degrees of adiposity in the HIV+ group, whereas this was not the case for the controls. Similar results were obtained for upper SAT as a % of total SAT (data not shown).

The relations of AT compartment sizes and AT distribution with $S_I$ are shown in the figures. Specifically, the relations of $S_I$—adjusted for age, weight, and height—with total SAT are shown in Figure 1, with VAT are shown in Figure 2, with IMAT are shown in Figure 3, and with VAT:SAT are shown in Figure 5. Analyses were also performed for upper SAT as a percentage of total SAT and for the absolute values of leg SAT and upper SAT, but no additional information was obtained. We computed the residual values of $S_I$ in a general linear model after adjustment for age, height, and weight. Because $S_I$ was measured with 2 different methods (using insulin for the HIV+ group and tobutamides for those in control group), a group factor (HIV+ group, control group 1, and control group 2) was initially entered in the analyses. Because no interactions between the control groups were observed, the results are shown for the control groups combined, with HIV status (positive or negative) entered as a factor in the model. We determined whether any of the AT measures significantly contributed to the additional variance in $S_I$ after these adjustments and whether significant interactions were observed in these relations between the HIV+ and healthy controls.

A significant interaction by HIV status was observed for the relations of total SAT with residual $S_I$ (after adjustment for age, height, and weight; Figure 1; $P < 0.001$ for slope interaction). For total SAT, a positive relation was observed in the HIV+ women (Figure 1; $r = 0.64, P = 0.006$), whereas the relation in

**FIGURE 1.** Relation between residual insulin sensitivity ($S_I$) and total subcutaneous adipose tissue (SAT) in HIV-positive (HIV+; △) and control (●) women. $S_I$ values were log transformed for normality. Residual $S_I$ is the difference between the observed and the expected values of $S_I$, calculated through multiple regression as a function of age, height, and weight. SAT was measured by whole-body magnetic resonance imaging. $P < 0.001$ for interaction of slopes by HIV status. $r = 0.64 (P = 0.006)$ for HIV+ women and $r = -0.22 (P = 0.2)$ for control women.

**FIGURE 2.** Relation between residual insulin sensitivity ($S_I$) and visceral adipose tissue (VAT) in HIV-positive (HIV+; △) and control (●) women. $S_I$ values were log transformed for normality. Residual $S_I$ is the difference between the observed and the expected values of $S_I$, calculated through multiple regression as a function of age, height, and weight. VAT was measured by whole-body magnetic resonance imaging. $P = 0.54$ for interaction of slopes by HIV status.

**FIGURE 3.** Relation between residual insulin sensitivity ($S_I$) and intermuscular adipose tissue (IMAT) in HIV-positive (HIV+; △) and control (●) women. $S_I$ values were log transformed for normality. Residual $S_I$ is the difference between the observed and the expected values of $S_I$, calculated through multiple regression as a function of age, height, and weight. IMAT was measured by whole-body magnetic resonance imaging. $P = 0.25$ for interaction of slopes by HIV status.
SAT with residual SI:

\( /L1152 \\

(HIV of visceral to subcutaneous adipose tissue (VAT:SAT) in HIV-positive women. The limb fat characterized in previous studies that used DXA included both leg IMAT and intramyocellular lipid in addition to SAT (32–35). We found a relative increase in IMAT and a relative decrease in leg SAT in our obese HIV+ women, which suggests that IMAT may have biologic characteristics different from those of SAT.

We also found that the relation between whole-body IMAT and insulin resistance did not differ significantly between the obese HIV+ group and the control group and that a high IMAT was independently associated with insulin resistance in both groups. These findings confirm our previous reports in HIV− women, which suggest that IMAT plays an important role in influencing insulin sensitivity independent of HIV status (28). Although reports regarding differences between VAT and SAT in HIV− individuals have been published (45), reports regarding the biologic characteristics of IMAT in HIV− or HIV+ are lacking (28, 38–39). The influence of IMAT on insulin action may be due to its proximity to the muscle cells, similar to the effects of intramyocellular lipids (27). However, relatively greater amounts of IMAT may also reflect other characteristics of whole-body AT, such as abnormalities in the largest storing depot, SAT. Further studies are clearly needed to characterize whole-body IMAT in both HIV+ and HIV− individuals.

We found that the association between absolute SAT accumulation and SAT was significantly different between the HIV+ and control groups. In contrast with results in the control group and with previous reports in HIV− obese women (4), the relation of absolute SAT accumulation with SI was positive in the HIV+ obese women in the present study. These findings were replicated even if absolute values of regional SAT (upper body or leg) were used (not shown). However, SAT distribution, i.e., a relatively low amount of leg SAT (expressed as a percentage of total SAT), was similarly related to a lower SI (insulin resistance) in both the obese HIV+ and the control groups. These relations were independent of VAT or IMAT. Differential characteristics of SAT in the upper versus the lower body could explain this finding. Gluteal and leg AT display increased glucose transport capacity, decreased sensitivity to lipolytic agents, and increased sensitivity to antilipolytic agents (46, 47)—in short, a higher capacity to store fat than the abdominal SAT. Less leg SAT could therefore reflect qualitative changes in overall SAT (more insulin resistance with decreased capacity to store), which could underlie the relation between SAT distribution and insulin resistance in both upper-body obese HIV− and HIV+ lipodystrophy. Indeed, recent data from 2 large studies in individuals not characterized by HIV status show associations between lower relative amounts of leg fat or thigh SAT and unfavorable glucose and lipid concentrations, independent of higher abdominal fat (36, 37).

We found VAT accumulation was not independently associated with insulin resistance in all groups of women studied. The relation between VAT and SI was negative albeit not significant (Figure 1; \( r = -0.22, P = 0.2 \)). No significant interactions by HIV status were found for the relations of VAT, IMAT, leg SAT%, and VAT:SAT with residual SI; \( P = 0.54 \) (Figure 2), \( P = 0.25 \) (Figure 3), \( P = 0.22 \) (Figure 4), and \( P = 0.08 \) (Figure 5) for slope interactions, respectively. For both groups combined, the best model predicting a low SI included significant contributions by both high IMAT and low leg SAT% (independent of age, weight, and height) and with no interaction between groups (overall \( r^2 = 0.44, P = 0.0003 \)). Neither VAT nor VAT:SAT was independently associated with SI in any of the models.

**DISCUSSION**

Associations between insulin resistance and both fat reduction and fat accumulation (7–9, 11–19) have been reported in previous cross-sectional studies in HIV+ individuals with lipodystrophy. Determining which aspect of fat distribution best represents increased insulin resistance in this HIV+ population is more difficult to assess in obese individuals, and a separate evaluation by sex is needed (34). Therefore, in this study we report the relation between AT distribution and insulin resistance in obese HIV+ women and in obese healthy controls. A unique aspect of our study was the measurement of whole-body IMAT and of SAT distribution, which allowed the separation of superficial SAT in the trunks and legs (upper-body SAT above the level of the greater trochanter and leg SAT below this level), independent of accumulation of IMAT, VAT, or other fat depots, such as intramyocellular lipid (28).

Consistent with previous reports, we found no decrease in the absolute amounts of AT but significant AT redistribution with relatively less SAT and more VAT and IMAT in the obese HIV+ women than in the controls (35). In previous studies that used DXA, higher absolute and relative amounts of trunk fat accumulation were found in HIV+ women with lipodystrophy (9–11, 32, 43), in contrast with lower absolute amounts of total body and lower relative amounts of limb fat in HIV+ men with lipodystrophy (10, 13, 14, 32, 44). By CT or MRI, the increase in trunk fat in HIV+ women was attributed to an increase in VAT (32–35). Although lower absolute amounts of SAT have not been reported in HIV+ women as they have been in men (13, 14, 33), a lower ratio of abdominal SAT to VAT area (13, 14, 32), which suggests fat redistribution, was found in both sexes. This is the first report to address IMAT measurements in obese HIV+ women. The limb fat characterized in previous studies that used DXA included both leg IMAT and intramyocellular lipid in addition to SAT (32–35). We found a relative increase in IMAT and a relative decrease in leg SAT in our obese HIV+ women, which suggests that IMAT may have biologic characteristics different from those of SAT.

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We found VAT accumulation was not independently associated with insulin resistance in all groups of women studied. The relation between VAT and SI was negative albeit not significant in the controls and positive in the HIV+ group but was not statistically different in the 2 groups; neither of the calculated slopes was statistically different from zero. Although HIV+ women with lipodystrophy were reported to have increased VAT and to be more insulin resistant than HIV+ women without lipodystrophy (9, 11, 32),

![FIGURE 5. Relation between residual insulin sensitivity (SI) and the ratio of visceral to subcutaneous adipose tissue (VAT:SAT) in HIV-positive (HIV+; △) and control (○) women. SI values were log transformed for normality. Residual SI is the difference between the observed and the expected values of SI, calculated through multiple regression as a function of age, height, and weight. VAT:SAT was measured by whole-body magnetic resonance imaging. \( P = 0.08 \) for interaction of slopes by HIV status.](image-url)
except for earlier studies in lean men and women (16, 30), most studies have not shown an independent relation between increased VAT and insulin resistance in HIV+ women (15, 26, 31). Therefore, our results in obese women could not be generalized to HIV+ men with lipodystrophy or to leaner HIV+ women. Still, it is of note that none of the previous studies made measurements of IMAT or SAT distribution.

Because HAART may directly alter both insulin sensitivity (48, 49) and AT metabolism (35, 50), it is not surprising that we found differences between the HIV+ and control groups in the relations of certain AT depots (ie, whole-body SAT) with insulin resistance; one could argue that such relations in HIV—lipodystrophy should not be compared with those in healthy obese controls. However, the similarities we found in the relations of IMAT and SAT distribution with insulin resistance in both obese HIV+ and obese controls suggest that a partly common pathophysiologic mechanism may underlie these relations for both groups. For example, the relation between low leg SAT% and insulin resistance in both the HIV lipodystrophy and control subjects may be a consequence of a relative decreased amount of protective SAT (leg SAT) in both groups. Loss of limb fat, specifically of SAT [whereas intramyocellular fat (27) and probably IMAT are spared], has been attributed to HAART in longitudinal studies (51, 52). The heterogeneity of HAART in our HIV+ subjects and the small sample size precludes us from determining any specific effect of an antiretroviral drug class on insulin resistance and fat redistribution. Longitudinal studies are needed to further clarify these relations.

Finally, we acknowledge that the population of HIV+ women that we studied was small and self selected for the desire to lose weight, making generalizations to the larger HIV+ population was difficult. Nevertheless, similarities of findings in these obese HIV+ women as in reasonably representative healthy controls decrease the risk of such findings being biased by the selection of the study’s HIV+ cohort. In addition, the attributes of our population highlight the importance of our results because the women we studied were mostly African American and Hispanic; these women tend to be overweight or obese and tend to remain obese when infected with HIV (21, 22). Our study needs to be extended to other HIV-infected individuals, specifically men and HIV+ women with a larger range of body weight.

In conclusion, aspects of AT distribution associated with insulin resistance in obese HIV+ women seeking weight loss are a high whole-body IMAT and a low leg SAT distribution. Clinically recognition of these aspects of AT distribution may be difficult and important for studies of interventions aimed at improving insulin resistance in obese HIV+ women.

We thank our study participants and the staff members of Columbia General Clinical Research Center, NY Obesity Research Center (Metabolism Core and Body Composition Core Labs) and the GI Immunology Department, St Luke’s Roosevelt Hospital Center, St Luke’s Roosevelt Hospital Center.

The authors’ responsibilities were as follows—JBA, DPK, and ESE: study design; JBA, SK MW, ESB, and ESE: subject recruitment and data collection; QH: MRI analyses; JBA and SH: data analysis; SH: statistical expertise; JBA, DPK, and ESE: critical review of the manuscript for intellectual content. JBA, DPK, and ESE received honoraria and grant support from Bristol-Myers Squibb for HIV lipodystrophy-related studies and educational activities. None of the authors had a conflict of interest to disclose.

REFERENCES

A low-glycemic-load diet improves symptoms in acne vulgaris patients: a randomized controlled trial

Robyn N Smith, Neil J Mann, Anna Braue, Henna Mäkeläinen, and George A Varigos

ABSTRACT

Background: Although the pathogenesis of acne is currently unknown, recent epidemiologic studies of non-Westernized populations suggest that dietary factors, including the glycemic load, may be involved.

Objective: The objective was to determine whether a low-glycemic-load diet improves acne lesion counts in young males.

Design: Forty-three male acne patients aged 15-25 y were recruited for a 12-wk, parallel design, dietary intervention incorporating investigator-blinded dermatology assessments. The experimental treatment was a low-glycemic-load diet composed of 25% energy from protein and 45% from low-glycemic-index carbohydrates. In contrast, the control situation emphasized carbohydrate-dense foods without reference to the glycemic index. Acne lesion counts and severity were assessed during monthly visits, and insulin sensitivity (using the homeostasis model assessment) was measured at baseline and 12 wk.

Results: At 12 wk, mean (±SEM) total lesion counts had decreased more (P = 0.03) in the low-glycemic-load group (−23.5 ± 3.9) than in the control group (−12.0 ± 3.5). The experimental diet also resulted in a greater reduction in weight (−2.9 ± 0.8 kg; P < 0.001) and body mass index (in kg/m²; −0.92 ± 0.25 compared with 0.01 ± 0.11; P = 0.001) and a greater improvement in insulin sensitivity (−0.22 ± 0.12 compared with 0.47 ± 0.31; P = 0.026) than did the control diet.

Conclusion: The improvement in acne and insulin sensitivity after a low-glycemic-load diet suggests that nutrition-related lifestyle factors may play a role in the pathogenesis of acne. However, further studies are needed to isolate the independent effects of weight loss and dietary intervention and to further elucidate the underlying pathophysiologic mechanisms. Am J Clin Nutr 2007;86:107-15.

KEY WORDS Acne, glycemic index, glycemic load, insulin resistance, hyperinsulinemia

INTRODUCTION

Acne is a common and complex skin disease that affects individuals of all ages. In Western populations, acne is estimated to affect 79–95% of the adolescent population, 40–54% of individuals older than 25 y, and 12% of women and 3% of men by middle age (1). In contrast, acne remains rare in non-Westernized societies such as the Inuit (2), Okinawan Islanders (3), Ache hunter-gatherers, and Kitavan Islanders (1). Although familial and ethnic factors are implicated in acne prevalence, this observation is complicated by the finding that incidence rates of acne have increased with the adoption of Western lifestyles (2). These observations suggest that lifestyle factors, including diet, may be involved in acne pathogenesis.

Historically, much debate has surrounded the subject of diet in the management of acne. In the 1930s, acne was considered to be a disease of disturbed carbohydrate metabolism because early work suggested that impaired glucose tolerance occurred in acne patients (4). On the basis of these observations and the anecdotal impressions of physicians, patients were often discouraged from eating excessive amounts of carbohydrates and high-sugar foods (5, 6). The diet and acne connection finally fell from favor in 1969 when a clinical study found no exacerbation of acne lesions in a group that ingested a chocolate bar compared with a group that ingested a placebo bar (7). Although it is the most widely cited reference dissociating diet and acne, this study has been criticized for a number of design flaws, including the similar nutrient composition of the placebo and the chocolate bar (8–10).

Recently, there has been a reappraisal of the diet and acne connection because of a greater understanding of how diet may affect endocrine factors involved in acne (1, 10). Of interest is the concept of the glycemic index (GI)—a system of classifying the glycemic response of carbohydrates. Because the GI can only be used to compare foods of equal carbohydrate content, the glycemic load was later developed to characterize the glycemic effect of whole meals or diets (GI × available dietary carbohydrate). Cordain et al (1) postulated that high-glycemic-load diets may be a significant contributor to the high prevalence of acne seen in Western countries. The authors speculate that the frequent consumption of high-GI carbohydrates may repeatedly expose adolescents to acute hyperinsulinemia. Hyperinsulinemia has been implicated in acne pathophysiology because of its association with increased androgen bioavailability and free concentrations of insulin-like growth factor I (IGF-I) (10, 11). Therefore, we...
hypothesized that low-glycemic-load dietary interventions may have a therapeutic effect on acne based on the beneficial endocrine effects of these diets. Consequently, the aim of this preliminary study was to investigate the efficacy of a low-glycemic-load diet in reducing the severity of acne symptoms.

SUBJECTS AND METHODS

Subjects

Males with facial acne were recruited through posted fliers at the RMIT University (Melbourne, Australia) and newspaper advertisements. Informed consent was obtained from each participant or guardian (if aged <18 y), and the study was conducted at RMIT University after obtaining approval from the RMIT Human Ethics committee. This study included only male participants aged 15-25 y with mild-to-moderate facial acne. Participants were required to have had acne for >6 mo before recruitment. Individuals were excluded if they were currently taking medications known to affect acne or glucose metabolism. Additionally, a washout period of 6 mo was required for subjects who had previously taken oral retinoids or 2 mo for subjects who had taken oral antibiotics or topical antibacterial or retinoid agents.

Study design

It was calculated that 19 subjects per group would provide 80% power (at the 2-sided 5% level) to detect a difference of 20% in the reduction of acne lesions between groups, assuming an SD of 22%. To compensate for subject withdrawal, 54 subjects were enrolled in the study.

Eligible participants were recruited between June 2003 and June 2004. Approximately 2–3 wk after recruitment, participants attended their baseline appointment and were randomly assigned to either the low-glycemic-load (LGL) or the control group (Figure 1). Randomization was carried out by computer generated random numbers, and allocation to groups was performed by a third party.

This study was designed as a parallel dietary intervention study with investigator-blinded dermatology assessments. Topical therapy, in the form of a noncomedogenic cleanser, was standardized for both groups, and facial acne was scored at monthly visits (weeks 0, 4, 8, and 12) at the academic research clinic. On all visits, height and weight were measured. All subjects were weighed in light clothes, and body mass index (BMI) was calculated as weight (kg)/height squared (m). At baseline and 12-wk, a venous blood sample was taken after an overnight fast, and an oral glucose insulin sensitivity (OGIS) test was performed in a subgroup of participants from the LGL and control groups.

The primary endpoints of the study were changes in inflammatory lesion counts (papules, pustules, and nodules) and total lesion counts (inflammatory lesions and noninflammatory lesions) after 12 wk. Secondary endpoints included changes in anthropometric measures and insulin sensitivity indexes.

Dietary intervention

Participants were informed that the study’s intent was to compare the dietary carbohydrate to protein ratio and were not informed of the study’s true intent. The LGL diet was achieved by modifying the amount and type of carbohydrate. The LGL group was instructed to substitute high-GI foods with foods higher in protein (eg, lean meat, poultry, or fish) or with foods with a lower GI (eg, whole-grain bread, pasta, and fruit). Some staple foods were supplied, and the participants were urged to consume these or similar foods daily. Each participant received individualized dietary plans that were isocalorically matched with their baseline diet as determined from 7-d weighed and measured food records. The recommended LGL diet consisted of 25% of energy from protein, 45% from low-GI carbohydrates, and 30% energy from fats. In contrast, the control group received carbohydrate-dense staples and were instructed to eat these or similar foods daily. The foods provided had moderate-to-high GI values and were typical of their normal diet as evidenced from 7-d weighed and measured food records. The control group was not informed about the GI,
but were urged to include carbohydrates as a regular part of their diet. All participants were instructed on how to use food scales and to keep food records. During the study period, nutrient intakes were calculated from 3-d weighed and measured food records each month by using Australia-specific dietary analysis software (FOODWORKS; Xyris Software, Highgate Hill, Australia). Dietary compliance was monitored via regular telephone interviews, assessments of daily glycemic load, and 24-h urine samples (weeks 0 and 12) for an assessment of urea excretion relative to urinary creatinine as a marker of protein intake.

Calculation of dietary glycemic index and glycemic load

Daily dietary glycemic index and glycemic load were calculated from diet records. The dietary GI was calculated as \( \Sigma (GI \times \text{carbohydrate content in grams/100}) \), and the glycemic load was calculated as \( \Sigma (GI \times \text{carbohydrate content in grams/100}) \). The GI values used had glucose as the reference food and were taken from reference tables (12) and from Sydney University’s GI website (13). If a food from Australia was not available, the GI was estimated by using similar foods of known value.

Standardized topical lotion

All participants were provided with a topical cleanser (Cetaphil gentle skin cleanser; Galderma, Frenchs Forrest, Australia) and advised to use it in place of their normal wash, soap, or cleanser. The subjects began using the topical wash 2 wk before baseline and were asked to maintain a standard level of use during the trial.

Dermatology assessment

Scaling of the acne was performed by a dermatology registrar who was blinded to the group assignment of the participants. The registrar assessed facial acne occurrence and severity only using a modified Cunliffe-Leeds lesion count technique (14). To ensure that all acne lesions were counted, located, and graded by size and severity, lesions were mapped by placing a transparent plastic film with a laser-printed grid gently against the skin. Facial anatomical landmarks, such as the ear, chin, and tip of the nose, were used to ensure consistency between assessments. Each side of the face was assessed separately. Where necessary, the registrar palpated the skin to determine the lesion type. To maintain the reproducibility of this procedure, one physician performed all the dermatology assessments. A small group of volunteers (\( n = 4 \)) was counted 1-wk apart to evaluate the reproducibility by the same physician (9.5% CV).

Laboratory analyses

Code-labeled serum samples were stored at \(-80 \, ^\circ C\) for analysis after the study by an independent laboratory. Baseline and 12-wk samples for each participant were included in the same assay run to avoid interassay variability. Serum insulin was measured by using a commercially available microparticle enzyme immunoassay (Abbott Laboratories, Tokyo, Japan; intraassay CV: 4.0%). Capillary blood glucose was measured on the day of testing with a Glucose 201+ analyzer (HemoCue, Sweden; intraassay CV: 1.6%).

Insulin sensitivity measures

The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting glucose (mmol/L) \( \times \) fasting insulin (\( \mu U/mL \)/22.5 (15). We also used the model-derived formula proposed by Mari et al (16) to calculate the OGIS index from a 2-h oral-glucose-tolerance test. This formula was calculated using 6 fixed-rate constants: oral glucose dose (75 g); body surface area; glucose concentrations (mg/dL) at 0, 90, and 120 min; and insulin concentrations (\( \mu U/mL \)) at 0 and 90 min. In the present study, some participants were unable to complete the 2-h test because of scheduling interference (eg, school and work commitments). Consequently, the OGIS data presented are for only 18 subjects in the LGL group and for 17 subjects in the control group.

Statistical analysis

All statistical analyses were performed with the use of SPSS 11.0 for WINDOWS (SPSS Inc, Chicago, IL). Baseline characteristics were analyzed for between-group significance with a Mann-Whitney \( U \) test or an independent-sample \( t \) test, depending on whether or not the data were normally distributed. Repeated-measures analysis of variance was used to analyze dietary and acne (lesion count) data and to explore the effects of time, treatment, and an interaction of these 2 factors. We compared changes in lesion counts using repeated-measures analysis of log-transformed data at each follow-up visit with baseline lesion counts as the covariate. \( P \) values <0.05 were considered significant. Analysis of covariance (ANCOVA) was used to test for overall treatment differences at 12 wk, with baseline data as the covariate. Secondary analyses were performed with adjustment for changes in BMI.

The primary clinical outcome (changes in lesion counts at 12 wk) was analyzed by using an intention-to-treat model for all randomized subjects with the use of the last measurement carried forward for all missing data.

Bivariate linear regression analysis was also conducted, pooling data from both groups, to explore relations between dietary variables and acne improvement.

RESULTS

Subjects

Forty-three subjects completed the study per protocol (Figure 1). Seven participants did not complete the study (5 in the control and 2 in the LGL group), and 4 were removed from data set (2 began taking acne medications and 2 were noncompliant). Baseline characteristics of the subjects are shown in Table 1.

Dietary intakes

Dietary intakes of the LGL and control groups at baseline and during the trial period is shown in Table 2. No significant group differences were observed in any of the dietary variables at baseline. During the trial period, dietary glycemic load decreased significantly in the LGL group compared with the control group, and this change was achieved by a reduction in carbohydrate intake and by the consumption of low-GI foods (as indicated by a reduction in the calculated dietary GI). Protein intake increased in the LGL group and decreased slightly in the control group, which indicates that some carbohydrates were replaced with...
TABLE 1
Subject characteristics at baseline by dietary group

<table>
<thead>
<tr>
<th>Variable</th>
<th>LGL group (n = 23)</th>
<th>Control group (n = 20)</th>
<th>p²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethnicity (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>20</td>
<td>17</td>
<td>0.60</td>
</tr>
<tr>
<td>Asian</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>18.2 ± 0.5</td>
<td>18.5 ± 0.5</td>
<td>0.76</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>73.5 ± 2.5</td>
<td>73.3 ± 3.3</td>
<td>0.90</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.9 ± 0.6</td>
<td>22.5 ± 0.7</td>
<td>0.34</td>
</tr>
<tr>
<td>Total lesion count</td>
<td>40.6 ± 5.0</td>
<td>34.9 ± 4.3</td>
<td>0.40</td>
</tr>
<tr>
<td>Inflammatory lesion count</td>
<td>31.9 ± 3.9</td>
<td>28.4 ± 3.6</td>
<td>0.72</td>
</tr>
<tr>
<td>OGIS (mL·m⁻³·min⁻¹)</td>
<td>481 ± 9</td>
<td>503 ± 9</td>
<td>0.12</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.6 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>0.88</td>
</tr>
</tbody>
</table>

OGIS, oral glucose insulin sensitivity; HOMA-IR, homeostasis model assessment of insulin resistance; LGL, low glycemic load.

Studies outcomes

As shown in Figure 2, both the LGL and control groups showed reductions in inflammatory and total lesion counts from 0 to 12 wk. Repeated-measures analysis that used baseline counts as the covariate indicated an overall difference between the LGL and control groups (main effect of group), but no significant change over time (main effect of time) or difference in the time course between the groups (group × time interaction) was observed. At 12 wk, the LGL group had a greater reduction in the mean number of total and inflammatory lesions than did the control group (Table 3). Examples of acne improvement in the LGL group are shown in Figure 3. The mean number of total lesions fell by 23.5 (51%) in the LGL group and by 12.0 (31%) in the control group (P = 0.03). Inflammatory lesion counts fell by 17.0 (45%) in the LGL group and by 7.4 (23%) in the control group (P = 0.02). The results at 12 wk were also materially unchanged by an intention-to-treat analysis.

The LGL group also showed significant reductions in weight (P = 0.001), BMI (P = 0.001) and HOMA-IR (P = 0.026) when compared with the control group. The change in HOMA correlated compared with the change in OGIS index (r = −0.36, P = 0.035), with both models suggesting a trend for improved insulin sensitivity in the LGL group and a trend for increasing insulin resistance in the control group. Statistical adjustment of study endpoints for the change in BMI was found to alter the outcome for HOMA-IR (P = 0.10) and total lesion counts (P = 0.07), but not inflammatory

TABLE 2
Dietary intakes of low-glycemic-load (LGL) and control groups at baseline and during the trial period

<table>
<thead>
<tr>
<th>Variable</th>
<th>LGL group (n = 23)</th>
<th>Control group (n = 20)</th>
<th>p²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ/d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>10 625 ± 572</td>
<td>10 540 ± 546</td>
<td></td>
</tr>
<tr>
<td>Trial period</td>
<td>9320 ± 460</td>
<td>10 620 ± 494</td>
<td></td>
</tr>
<tr>
<td>Dietary glycemic index</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>57.5 ± 1.0</td>
<td>57.2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Trial period</td>
<td>43.2 ± 0.8</td>
<td>56.4 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Dietary glycemic load</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>174.7 ± 9.1</td>
<td>181.5 ± 11.5</td>
<td></td>
</tr>
<tr>
<td>Trial period</td>
<td>101.5 ± 6.1</td>
<td>174.3 ± 10.7</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate (% of total energy)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>50.2 ± 1.1</td>
<td>48.2 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Trial period</td>
<td>44.1 ± 1.3</td>
<td>50.1 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Protein (% total energy)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>16.3 ± 0.6</td>
<td>17.5 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Trial period</td>
<td>22.7 ± 0.8</td>
<td>17.4 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Total fat (% total energy)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>32.5 ± 1.1</td>
<td>31.8 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Trial period</td>
<td>31.5 ± 0.9</td>
<td>31.4 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Saturated fat (% total energy)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>13.5 ± 0.6</td>
<td>12.9 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Trial period</td>
<td>9.0 ± 0.4</td>
<td>13.0 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Fiber (g/d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>25.3 ± 1.8</td>
<td>25.2 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>Trial period</td>
<td>36.9 ± 2.0</td>
<td>25.2 ± 2.0</td>
<td></td>
</tr>
</tbody>
</table>

1 Repeated-measures ANOVA was done to incorporate data from all time points and to evaluate the differences between the LGL and the control groups (main effect of group), the change over time (main effect of time), and the differences in the time course between the 2 groups (group × time interaction).
2 An independent-sample t test showed no significant differences between the LGL and the control groups for any of the listed dietary variables at baseline.
3 x ± SE (all such values).
4 Means of data collected at 4, 8, and 12 wk.
counts ($P = 0.04$). However, we found no significant interaction effect of dietary treatment and the change in BMI on acne lesion counts.

Dietary variables as predictors of acne improvement

Dietary correlates with acne improvement include reductions in glycemic load ($r = 0.49$, $P = 0.001$), dietary GI ($r = 0.30$, $P = 0.05$), carbohydrate intake ($r = 0.46$, $P = 0.002$), percentage saturated fat ($r = 0.36$, $P = 0.019$), and total energy intake ($r = 0.40$, $P = 0.010$). These relations are shown in Figure 4.

DISCUSSION

This study was the first randomized controlled intervention to examine the influence of dietary glycemic load on the clinical assessment of acne. After adjustment for differences in acne severity at baseline, we found that the LGL group had greater overall reduction in total and inflammatory lesion counts than did the control group. Analysis of the primary endpoint data also showed that the differences between groups remained significant after an intention-to-treat model was used. However, we found no significant effect of time on acne, possibly because acne is a
dynamic condition in which both spontaneous improvements and flares can occur over time.

Few well-controlled dietary studies have examined the effect of diet on acne. Fulton et al (7), in a crossover single-blind study, found no effect of chocolate on acne when compared with a placebo bar. However, a later examination of the ingredients in the placebo bar indicated that the fatty acid composition and sugar contents were virtually identical to that found in the chocolate (8). Anderson examined the effect of the daily consumption of chocolate, milk, or nuts and found no effect on acne (17). However, this study has also been criticized for its small sample size, short follow-up, and lack of control (18). Chiu et al (19) showed, in university students, an association between worsening diet quality and exacerbation of acne during a preexamination period. However, stress was found to be the main contributing factor, and diet was assessed by using a nonquantitative, self-assessed measure of diet quality. Recently, a retrospective evaluation of dietary intake showed a positive association between milk intake and physician-diagnosed severe acne (20). However, the authors admit that this association may have been affected by the imprecision inherent in the measurement of dietary intakes via dietary recall.

High-glycemic-load diets have recently been implicated in acne etiology because of their ability to increase the insulin demand and other factors associated with insulin resistance (eg, hyperphagia, elevated nonesterified fatty acids, and obesity) (11). Clinical and experimental evidence suggests that insulin may increase androgen production and affect, through its influence on steroidogenic enzymes (21), gonadotrophin releasing hormone secretion (22) and sex hormone–binding globulin production (23–25). Additionally, insulin has been shown to decrease a binding protein for IGF-I, which may facilitate the effect of IGF-I on cell proliferation (26). Overall, these events may influence 1 or more of the 4 underlying causes of acne: 1) increased proliferation of basal keratinocytes within the pilosebaceous duct, 2) abnormal desquamation of follicular cornocytes, 3) androgen-mediated increases in sebum production, and 4) colonization and inflammation of the comedo by Propionibacterium acnes (11).

The role of insulin in acne development is also supported by the high prevalence of acne in women with polycystic ovary syndrome (PCOS), a condition associated with insulin resistance, hyperinsulinemia, and hyperandrogenism (27). Insulin resistance is believed to be the underlying disturbance in PCOS, because it generally precedes and gives rise to the cluster of endocrine abnormalities that characterize PCOS (elevated androgen and IGF-I concentrations and low sex hormone–binding globulin; 28). Treatments for PCOS now include oral hypoglycemic agents, which improve insulin sensitivity, restore fertility, and alleviate acne (29).

Our study also suggests that changes in acne may be closely related to changes in insulin sensitivity, because we observed a positive effect of the LGL diet on insulin sensitivity compared with the control diet. However, the improvement in insulin sensitivity may be attributable not only to the reduction in glycemic load (30), but also to the reduction in body mass. The participants in the LGL group lost weight despite receiving dietary advice to maintain their baseline energy intake. This may have been due to the dual effect of added protein and low-GI foods, because both influence hunger and satiety. Feeding studies have shown that low-GI foods increase satiety, delay hunger, and decrease food intake when compared with high-GI foods (31, 32). Similar effects on satiety have been reported for high-protein meals compared with isocaloric high-carbohydrate or high-fat meals (33). Therefore, the combined effect of low-GI foods and added protein may have reduced ad libitum food intake, which made it difficult for our participants to maintain the energy density of their baseline diets. This observation is supported by previous studies that showed ad libitum LGL diets to reduce energy intake without the need for an externally imposed energy restriction (34, 35).

Because the participants in the LGL group lost weight, we cannot preclude a role for the change in BMI in the overall treatment effect. When we statistically adjusted the data for changes in BMI, the effect of the LGL diet on total lesion counts and HOMA-IR was lost. This suggests that the therapeutic effect may be a factor of the weight loss or simply that weight loss is another manifestation of an LGL diet. Apart from women with PCOS, little evidence suggests an association between acne severity and body weight. Aizawa and Niimura (36) showed mild peripheral insulin resistance in female acne sufferers that was not associated with obesity or menstrual irregularities. In contrast, Bourne and Jacobs (29) showed that adult men with acne were
significantly heavier (5.6 kg) than men without acne. However, the authors showed that this association was dependent on age, because weight was not associated with acne in adolescents aged 15-19 y. Our data also showed a significant correlation between acne lesion counts and BMI in males aged 18-25 y, but this was not true for the subjects aged <18 y (data not shown). The reason for this observation is unknown, but it is possible that the transient decline in insulin sensitivity that occurs with the progression through puberty may trigger acne in the younger population (37, 38).

A few limitations of the study should be addressed. First, it is possible that the topical application of the mild skin cleanser may have contributed to the acne improvement through effects on the epidermal barrier function (39). Because acne improved in the control group without any significant changes to their diet, a possible direct effect of the cleanser should be considered. Second, because of the nature of the LGL dietary intervention, we cannot solely attribute the treatment effects to changes in glycemic load because other dietary factors (eg, zinc and vitamin A intake) may mediate or confound the relation between diet and acne improvement. Last, this study relied on self-reporting of dietary intakes. Underreporting the quantity of food eaten is a known source of measurement error when assessing adolescent diets (40).

To our knowledge, this is the first study to show a therapeutic effect of dietary intervention on acne. After 12 wk, the LGL diet was shown to significantly reduce acne lesion counts and improve insulin sensitivity when compared with a high-glycemic-load diet. Although we could not isolate the effect of the LGL diet from that of

FIGURE 3. Photographs of acne improvement in the low-glycemic-load group. A and B: subject A at baseline and 12 wk respectively; C and D: subject B at baseline and 12 wk, respectively; and E and F: subject C at baseline and 12 wk, respectively.
weight loss, these findings are consistent with earlier suggestions of
the association between hyperinsulinemia and acne. These ob-
servations will need to be substantiated and the underlying mech-
asisms determined in larger-scale studies.

The authors’ responsibilities were as follows—RNS, NJM, and AB: con-
tributed to the writing of this manuscript; RNS, AB, and HM: helped with the
data collection; RNS: responsible for the data analysis; and NJM and GAV:
provided significant advice and supervised the project. The sponsor of this
study, Meat and Livestock Australia, had no role in the data collection, data
analysis, data interpretation, or submission of this article for publication.
RNS received a postgraduate scholarship from MINTRAC (National Meat
Industry Training Council of Australia).

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A LOW-GLYCEMIC-LOAD DIET AND ACNE VULGARIS

Sugars and satiety: does the type of sweetener make a difference?1–3

Pablo Monsivais, Martine M Perrigue, and Adam Drewnowski

ABSTRACT

Background: Widespread use of high-fructose corn syrup (HFCS) in beverages has been linked to rising obesity rates. One hypothesis is that HFCS in beverages has little satiating power.

Objective: The objective of the study was to compare the relative effect of commercial beverages containing sucrose or HFCS on hunger, satiety, and energy intakes at the next meal with the use of a within-subject design.

Design: Thirty-seven volunteers (19 men, 18 women) aged 20–29 y consumed isocaloric cola beverages (215 kcal) sweetened with sucrose, HFCS 42, or HFCS 55. HFCS 42 contains 42% fructose, and HFCS 55 contains 55% fructose. Diet cola (2 kcal), 1%-fat milk (215 kcal), and no beverage were the control conditions. The 5 beverages were consumed at 1010 (2 h after a standard breakfast). Participants rated hunger, thirst, and satiety at baseline and at 20-min intervals after ingestion. A tray lunch (1708 kcal) was served at 1230, and energy intakes were measured. The free sugars content of sucrose-sweetened cola was assayed at the time of the study.

Results: We found no differences between sucrose- and HFCS-sweetened colas in perceived sweetness, hunger and satiety profiles, or energy intakes at lunch. The 4 caloric beverages tended to partially suppress energy intakes at lunch, whereas the no-beverage and diet beverage conditions did not; the effect was significant ($P < 0.05$) only for 1%-fat milk. Energy intakes in the diet cola and the no-beverage conditions did not differ significantly.

Conclusion: There was no evidence that commercial cola beverages sweetened with either sucrose or HFCS have significantly different effects on hunger, satiety, or short-term energy intakes. Am J Clin Nutr 2007;86:116–23.

KEY WORDS Beverages, sucrose, fructose, high-fructose corn syrup, HFCS, sweetness, hunger, fullness, satiety, energy intakes

INTRODUCTION

The introduction of corn sweeteners into the US food supply is said to have contributed to the current obesity epidemic (1–3). High-fructose corn syrup (HFCs) began to replace sucrose in soft drinks at approximately the same time that obesity rates in the United States began their sharp increase (2, 4). However, temporal parallels between HFCS consumption patterns and body-weight trends are not sufficient to show causality. Obesity has also increased sharply in countries where beverage consumption is lower than in the United States and HFCS is not a common sweetener (5).

One of the criteria for establishing causality in evidence-based medicine is a biologically plausible mechanism. Attempts to establish a causal link between soft drink consumption and rising obesity rates have therefore relied on the notion that caloric beverages in general (6), and HFCS-sweetened beverages in particular (2, 7), lack satiating power. Research reports have suggested that liquids were less satiating than were solids (8); that sugars were less satiating than was either protein or fat (9); and that HFCS blunted the satiety response more than did other sweeteners (2, 10, 11). The metabolic and endocrinologic processes associated with the ingestion of free fructose have featured prominently in arguments that HFCS-sweetened beverages are the principal culprit in the obesity epidemic (2, 6, 7).

However, satiety-related arguments based on the ingestion of pure fructose or fructose-rich stimuli (12, 13) may not apply to sweetened beverages, given that the 2 most common forms of HFCS—HFCS 55 and HFCS 42—contain 55% and 42% free fructose, respectively, and the remainder is free glucose. Furthermore, the sharp distinctions made between HFCS-sweetened and sucrose-sweetened beverages (2, 14) may be incorrect. The low pH of carbonated soft drinks favors the breakdown of sucrose into free glucose and free fructose before consumption (15), and the rate of hydrolysis is dependent on storage variables, temperature, and time (16). Perhaps most important, the short-term satiating power of foods and beverages may have little to do with the long-term regulation of body weight (13, 17).

The present study was a direct test of the hypothesis that HFCS-sweetened carbonated soft drinks differ significantly from sucrose-sweetened soft drinks and from low-fat milk in their effect on satiety. Aiming to approximate naturalistic conditions of soft drink use, we compared the effect of commercially available cola beverages, sweetened with sucrose or with 2 types of HFCS (HFCS 42 and HFCS 55), on hunger, satiety, and energy intakes (EIs) at the test meal. Because so much has been made of the metabolic differences between free fructose and fructose bound within disaccharide molecules (2, 14), we sent samples of the sucrose-sweetened beverage to be analyzed for free sugars content at the time of the experiment.

1 From the Nutritional Sciences Program, School of Public Health and Community Medicine (PM, MMP, and AD), and the Department of Dental Public Health Sciences, School of Dentistry (PM), University of Washington, Seattle, WA.

2 Supported by a grant from the American Beverage Association, by the Corn Refiners Association, and by fellowship T32 DE07132 from the National Institute of Dental and Craniofacial Research (to PM).

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**SUBJECTS AND METHODS**

**Participants**

Thirty-seven participants (19 M, 18 F) aged 20–29 y were recruited at the University of Washington with the use of advertisements and flyers. A telephone-administered screening interview was used to verify eligibility criteria. Eligible participants were normal-weight to overweight (body mass index (BMI; in kg/m²): 18–30], regularly consumed breakfast, did not smoke, and were not following a diet to gain or lose weight. Persons with food allergies or food restrictions; those who disliked ≥2 of the foods or beverages in the study; those taking prescription medications that were likely to affect taste, smell, or appetite; athletes in training; pregnant or lactating women; and persons reporting recent weight loss or weight cycling were excluded. Potential candidates were invited to report to the laboratory for a brief session, during which their weight and height were measured. The Eating Disorder Inventory (18) and the cognitive restraint subscale of the Eating Inventory (19) were administered as screening instruments to exclude persons with indications of eating disorders or restrained eating patterns. Persons who met all eligibility criteria were invited to participate and were given a reminder card stating the dates and times for the study sessions. To minimize variability, each participant was asked to report to the laboratory on the same day of the week throughout the study, to keep evening meals and activity levels on the day before each test as similar as possible, to refrain from drinking alcohol the day before each test, and to have a standardized breakfast at ≈0800 on the mornings when they were scheduled to have a test. The participants’ standardized breakfasts were consumed at home and were composed of specified servings of hot or cold cereals with milk along with a medium-sized apple, orange, or banana or a specified serving of low-fat yogurt along with a medium-sized fruit.

All participants provided written informed consent. The study protocol was approved by the Institutional Review Board at the University of Washington. All 37 subjects completed the study protocol and were compensated for their time.

**Study design**

The study followed a repeated-measures within-subject design, in which each participant returned for 6 separate test sessions. The sessions lasted from 0930 to 1310 and were spaced at least a week apart. The order of presentation of the 5 preloads and the no-beverage condition was counterbalanced. The same lunch foods were offered on all 6 testing occasions. The magnitude of the energy manipulation (0 or 215 kcal) was based on a review of previous studies in this area (20). Power analysis indicated that, with a power of 80% and an alpha of 0.05, a sample of 35 subjects differed in sugar composition. To keep both sweetness and energy constant, the preload volume was allowed to vary within narrow limits (from 475 to 525 mL.). The composition and energy density of the 5 beverage preloads are shown in Table 1. All beverages were coded and were served chilled, without ice, in opaque containers with a lid and a straw. Participants were asked to consume the entire amount within 15 min.

Samples of the sucrose-sweetened beverage were analyzed at regular intervals during the data collection phase. The analyses, conducted by the Analytic Chemistry Department of the Coca-Cola Company, used samples from the same production run (25 May 2005) of Coca-Cola Classic and were conducted at the same time as the data collection phase of the present study.

**Motivational ratings and hedonic evaluations**

Participants used computerized, semi-anchored visual analogue scales (VASs) to rate their hunger, fullness, thirst, nausea, and desire to eat. The VAS software was custom-written by using the LABVIEW graphic programming software (version 6.1; National Instruments, Austin, TX) that was running on 10 identical Macintosh G3 computers (Apple Computers, Cupertino, CA). Motivational scales were presented one at a time (ie, one scale per screen) on the computer monitor. Each participant used a mouse to position a cursor along the 100-mm bar displayed on a flat-panel LCD computer monitor. The VAS bars were anchored at each extreme with the labels “not at all . . .” and “extremely . . .” (22). A semi-anchored VAS was also used for quantifying several sensory and hedonic attributes of each beverage. Each sensory attribute scale also was anchored with the labels “not at all . . .” and “extremely . . .”. Hedonic ratings and ratings along 11 sensory attribute scales were obtained for each beverage.

**Table 1**

<table>
<thead>
<tr>
<th>Sugar composition</th>
<th>Sugars</th>
<th>Protein</th>
<th>Fat</th>
<th>Serving Energy</th>
<th>Energy density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preload</td>
<td>%/%</td>
<td>g</td>
<td>g</td>
<td>mL</td>
<td>kcal</td>
</tr>
<tr>
<td>HFCS 42</td>
<td>42/58</td>
<td>57.3</td>
<td>0.0</td>
<td>0.0</td>
<td>475</td>
</tr>
<tr>
<td>HFCS 55</td>
<td>55/45</td>
<td>57.7</td>
<td>0.0</td>
<td>0.0</td>
<td>525</td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartame</td>
<td>0/0</td>
<td>54.7</td>
<td>0.0</td>
<td>0.0</td>
<td>525</td>
</tr>
<tr>
<td>1%-fat Milk</td>
<td>0/50</td>
<td>27.2</td>
<td>16.7</td>
<td>5.2</td>
<td>495</td>
</tr>
</tbody>
</table>

1 HFCS 42 and HFCS 55, High-fructose corn syrup–sweetened cola containing 42% and 55% fructose, respectively; sucrose, sucrose-sweetened cola; aspartame, aspartame-sweetened cola. Data were from ESHA software (FOOD PROCESSOR version 8.1; Salem, OR) and manufacturers’ specifications.

2 Lactose in milk is a disaccharide made up of 1:1 glucose:galactose.

**Beverage stimuli**

The 5 beverages were cola sweetened with HFCS 42 (Sam’s Choice Cola; Cott Beverages, Toronto, Canada), cola sweetened with HFCS 55 (Coca-Cola Classic; Coca-Cola Co, Atlanta, GA), cola sweetened with sucrose (Coca-Cola Classic), cola sweetened with aspartame (Diet Coke; Coca-Cola Co), and 1%-fat milk (Darigold; Wesfarm Foods, Seattle, WA). All preload beverages with the exception of the diet cola (2 kcal) were isoenergetic (894 kJ or 215 kcal) and of comparable sweetness, but they differed in sugar composition. To keep both sweetness and energy constant, the preload volume was allowed to vary within narrow limits (from 475 to 525 mL.). The composition and energy density of the 5 beverage preloads are shown in Table 1.

**Test meal**

A lunch meal served on a tray was provided at 1230. Identical meals were provided on each occasion. The set meal was 7120 kJ (1708 kcal) and included a variety of foods, both savory and sweet. Each lunch consisted of a selection of 2 grains, 2 types of fruit, 2 vegetables, 2 cheeses, 2 meats, 2 candies, 1 yogurt, 1 ice cream cup, hummus, chips, and water. A large cup containing 591 mL (20 fl oz) still water was provided with the test lunch. Participants were told that they could have as much or as little as
within-subject effects were subject to Huynh-Feldt correction (aspartame, 1%-fat milk, and no beverage). Univariate tests of 42% HFCS, and 55% HFCS) and the 3 comparison conditions together and separately for the 3 sweetener conditions (sucrose, subjects factor. Analyses were conducted for all 6 conditions 2–8) as the within-subjects factors and sex as the between-ANOVA) with beverage type and time after ingestion (times Smirnov test (normal if Data analyses and statistical tests

We used SPSS for WINDOWS software [version 11.1 (23)] for all analyses. Normality was determined by the Kolmogorov-Smirnov test (normal if \( P > 0.05 \)). Analyses of motivational ratings used a nested repeated-measures analysis of variance (ANOVA) with beverage type and time after ingestion (times 2–8) as the within-subjects factors and sex as the between-subjects factor. Analyses were conducted for all 6 conditions together and separately for the 3 sweetener conditions (sucrose, 42% HFCS, and 55% HFCS) and the 3 comparison conditions (aspartame, 1%-fat milk, and no beverage). Univariate tests of within-subject effects were subject to Huynh-Feldt correction when the sphericity assumption was violated. Bonferroni-adjusted pairwise comparisons were made when ANOVAs were significant. Because there were no significant main effects or sex-related interactions (\( P > 0.05 \) for all tests), the data were combined by sex for each beverage condition. Analyses of EIs and the weight of foods and water consumed at lunch used a repeated-measures ANOVA with beverage type as the within-subject factor and sex as the between-subjects factor. The strength of the association between prelunch (time 8) appetite ratings and energy or water intakes at lunch was tested by using Pearson’s correlation coefficients. Prelunch appetite ratings were computed by averaging 3 proxies for appetite according to a method similar to that of Anderson et al (11): hunger, desire to eat, and the inverse of fullness (ie, 100 − fullness). Sweetness intensity and hedonic ratings were analyzed by repeated-measures ANOVA.

RESULTS

Participants and beverage stimuli

Mean (± SD) age was 22.6 ± 4.0 y for men and 23.4 ± 2.8 y for women. Mean body weight was 77.5 ± 10.7 kg for men and 60.2 ± 9.1 kg for women. Body mass index (BMI; in kg/m²) values were 23.4 ± 1.8 for men and 21.9 ± 2.7 for women. Analysis of sweetness ratings for the 4 cola beverages found a significant (\( F_{3, 99} = 11.1, P < 0.001 \)) main effect of beverage type. However, that was entirely due to the aspartame-sweetened cola, which was perceived as significantly less sweet than the 3 sugar-sweetened colas (\( P < 0.01 \) for all 3 comparisons). Cola beverages sweetened with sucrose, HFCS 55, and HFCS 42 did not differ significantly in perceived sweetness ratings. Milk (1%-fat) was not perceived as sweet. All 5 beverages (including milk) were rated as equally palatable by the participants. The analysis of hedonic preference ratings showed no significant main beverage effect (\( F_{41, 32} = 2.5 \)).

### Table 2

Energy and nutrient composition of foods provided at lunch

<table>
<thead>
<tr>
<th>Food</th>
<th>Carbohydrate</th>
<th>Protein</th>
<th>Fat</th>
<th>Sugar</th>
<th>Fiber</th>
<th>Portion</th>
<th>Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( g )</td>
<td>( g )</td>
<td>( g )</td>
<td>( g )</td>
<td>( g )</td>
<td></td>
<td>( kcal )</td>
</tr>
<tr>
<td>Reduced-fat crackers</td>
<td>21</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>7</td>
<td>7 pieces</td>
<td>120</td>
</tr>
<tr>
<td>Pita bread</td>
<td>36</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>1 piece</td>
<td>190</td>
</tr>
<tr>
<td>Banana, medium-sized</td>
<td>26.7</td>
<td>1.2</td>
<td>0.6</td>
<td>1.8</td>
<td>1</td>
<td>1 piece</td>
<td>105</td>
</tr>
<tr>
<td>Apple slices</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>1 bowl</td>
<td>35</td>
</tr>
<tr>
<td>Baby carrots</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>1 bowl</td>
<td>38</td>
</tr>
<tr>
<td>Sugar snap peas</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>1 bowl</td>
<td>40</td>
</tr>
<tr>
<td>Cheddar cheese</td>
<td>0</td>
<td>5</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>1 slice</td>
<td>90</td>
</tr>
<tr>
<td>Havarti cheese</td>
<td>0</td>
<td>6</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>1 slice</td>
<td>110</td>
</tr>
<tr>
<td>Yogurt (99% fat-free)</td>
<td>33</td>
<td>5</td>
<td>1.5</td>
<td>27</td>
<td>0</td>
<td>1 pack</td>
<td>170</td>
</tr>
<tr>
<td>Ham</td>
<td>2</td>
<td>10</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>2 slice</td>
<td>60</td>
</tr>
<tr>
<td>Hummus</td>
<td>24</td>
<td>8</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>8 tbsp</td>
<td>240</td>
</tr>
<tr>
<td>Chocolate candies</td>
<td>15</td>
<td>1</td>
<td>4.5</td>
<td>13</td>
<td>1</td>
<td>1 pack</td>
<td>100</td>
</tr>
<tr>
<td>Marshmallow snack bar</td>
<td>18</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>0</td>
<td>1 pack</td>
<td>90</td>
</tr>
<tr>
<td>Potato chips</td>
<td>12</td>
<td>1</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>1 pack</td>
<td>140</td>
</tr>
<tr>
<td>Vanilla ice cream and orange sherbet blend</td>
<td>15</td>
<td>1</td>
<td>3</td>
<td>13</td>
<td>0</td>
<td>1 pack</td>
<td>90</td>
</tr>
<tr>
<td>Totals</td>
<td>226.7</td>
<td>72.2</td>
<td>61.6</td>
<td>79</td>
<td>12.8</td>
<td>—</td>
<td>1708</td>
</tr>
</tbody>
</table>

1 Information was obtained from the food label or ESHA software (FOOD PROCESSOR version 8.1; Salem, OR).
Motivational ratings

All 5 beverages (caloric and not) led to lower hunger ratings during the initial 20 min after ingestion than were seen in the no-beverage condition. Repeated-measures ANOVA showed significant main effects of both beverage type and time and a significant beverage × time interaction (P < 0.001 for all). Separate analyses were then conducted for the 3 colas and the 3 comparison conditions.

The time course of energy and volume effects on short-term hunger is shown in Figure 1 (left). In the first 60 min after their ingestion, equal volumes of 1%-fat milk and noncaloric diet cola had comparable effects on perceived hunger. However, hunger ratings rose more rapidly in the diet cola condition than in the milk condition, and a separation in hunger ratings was visible before lunch. ANOVA found a significant (P < 0.001) main effect of beverage type and a significant (P < 0.005) beverage × time interaction.

In contrast, the 3 sugar-sweetened cola beverages, although different from the no-beverage condition, did not differ significantly from each other (Figure 1, right). There was no significant main beverage effect or beverage × time interaction.

ANOVA of fullness ratings found significant main effects of beverage type and time (P < 0.001 for both) and a significant beverage × time interaction (P < 0.005). As shown in Figure 2 (left), milk and diet cola had different effects on fullness ratings, depending on the time after ingestion. The beverage effect and the beverage × time interaction were significant (P < 0.01 for both). In contrast, the sucrose- and the HFCS-sweetened colas differed significantly (P < 0.001) from the no-beverage condition but not from each other. The beverage × time interaction was not significant (Figure 2, right).

Analysis of the desire-to-eat ratings found significant main effects of beverage type and time (P < 0.001 for both) and a significant beverage × time interaction (P < 0.01). It can be seen

FIGURE 1. Temporal profiles of mean hunger ratings as a function of preload condition for control beverages (left) and sugar-sweetened carbonated beverages (right). n = 37. VAS, visual analogue scale; HFCS 42 and 55, high-fructose corn syrup containing 42% and 55% fructose, respectively. The no-beverage control was associated with higher hunger levels than were the milk and aspartame-sweetened diet cola controls (P < 0.005), and the beverage × time interaction was significant (P < 0.005). Hunger ratings for the 3 sugar-sweetened beverages did not differ significantly.

FIGURE 2. Temporal profiles of mean fullness ratings as a function of preload condition for control beverages (left) and sugar-sweetened carbonated beverages (right). n = 37. VAS, visual analogue scale; HFCS 42 and 55, high-fructose corn syrup containing 42% and 55% fructose, respectively. The no-beverage control was associated with significantly lower fullness ratings than were the milk and aspartame-sweetened diet cola controls (P < 0.001 for both). Diet cola was associated with significantly lower fullness ratings than was milk (P < 0.05), and the beverage × time interaction was significant (P < 0.01). Fullness ratings for the 3 sugar-sweetened beverages did not differ significantly.
in Figure 3 (left) that 1%-fat milk and diet cola had different effects on the desire to eat, depending on the time after ingestion. The effect of beverage type was significant (P < 0.001), as was the beverage × time interaction (P < 0.05). Again, the sucrose- and the HFCS-sweetened colas did not differ significantly from each other (Figure 3, right). Analysis of thirst ratings (Figure 4) found significant main effects of beverage type and time (P < 0.001 for both). The beverage × time interaction also was significant (P < 0.01). Subjects in the no-beverage condition reported significantly higher thirst ratings than did those in the other 5 conditions (P < 0.05 for all comparisons). The 5 beverages did not differ significantly from each other in thirst ratings. Participant nausea ratings did not vary significantly by preload condition, and nausea did not vary significantly as a function of time.

Energy and nutrient intakes

For each condition, energy and water intakes at lunch, as well as the weight of all foods consumed, are shown in Table 3. Across all 6 conditions, men consumed an average of 1077 kcal at lunch, whereas women consumed an average of 862 kcal. Whereas this effect of sex on EI was significant (P < 0.005), the beverage type × sex interaction was not.

The 4 caloric beverages partially suppressed EI at lunch as compared with the EI in the no-beverage condition. The main effect of beverage type on EIs was significant (P < 0.05). However, pairwise comparisons showed that the effect was significant only for the 1%-fat milk preload (P < 0.05). EI s in the no-beverage condition and in the diet cola condition did not differ significantly, which indicated that preload volume had no effect on EIs by the time the test lunch was served (120 min after preload ingestion).

The combined energy content of the preload and the lunch also showed significant main effects of beverage type (P < 0.001). Pairwise comparisons showed that the 3 sugar conditions did not differ significantly from each other or from milk. However, all 4 caloric beverages differed significantly from both the diet cola and the no-beverage conditions.

Beverage type significantly affected the weight of food and the amount of water consumed at lunch (P < 0.001 for both). Participants in the no-beverage condition consumed significantly more water at lunch than did subjects in all of the other preload conditions except for the diet cola condition. The nutrients consumed in association with each beverage condition are shown in Table 4. Beverage type did not significantly affect the nutrient composition of the lunch meal. Overall, the meals selected and consumed by the participants provided 51.2% of energy as carbohydrate, 17.2% of energy as protein, and 32.8% of energy as fat. The nutrient composition of lunch did not differ significantly by sex.

Motivational ratings and energy intakes

A composite score of appetite was calculated by using the method of Anderson et al (11). The correlation between appetite ratings and EIs at lunch was significant for women (r = 0.33,
and 6.4% indicate concentrations of sucrose present in samples were free fructose and glucose. Dashed reference lines at 50.6% shaded bar), the principal sugars in the sucrose-sweetened cola during the time of the satiety study (period indicated by the factured (May 25). Free fructose increased from 32% to 44% of total sugars on June 30 to just above 45% of total sugar.

Free sugar content of sucrose-sweetened beverage

The progressive hydrolysis of sucrose in cola beverages over the course of the study is shown in Figure 5. Each point is based on the analysis of 3 samples. As sucrose hydrolyzed, its concentration declined from 36% of total sugars on June 30 to just above 10% on August 24, or ≈3 mo after the beverages were manufactured (May 25). Free fructose increased from 32% to ≈44%. Free glucose (not shown) followed the same course as fructose. During the time of the satiety study (period indicated by the shaded bar), the principal sugars in the sucrose-sweetened cola were free fructose and glucose. Dashed reference lines at 50.6% and 6.4% indicate concentrations of sucrose present in samples from a separate lot of sucrose-sweetened cola measured 10 d and 1 y after manufacture.

DISCUSSION

The argument that HFCS-sweetened beverages play a causal role in the obesity epidemic (24) rests, in part, on the notion that free fructose blunts the satiety response more strongly than do other sweeteners (2, 11, 13). Bray et al (14) made a sharp distinction between “free” and bound fructose in soft drinks, arguing further that HFCS-containing beverages could “never” have the same sweetness as sucrose-sweetened ones.

Free sugar content of sucrose-sweetened beverage

As the present study shows, the 3 cola beverages, which were sweetened with sucrose, HFCS 55, or HFCS 42, were perceived as equally sweet and significantly sweeter than diet cola. All 3 beverages showed identical temporal profiles of motivational ratings, which were different from the no-beverage condition. There were no differences between the reported temporal profiles for hunger, satiety, and the desire to eat obtained after the ingestion of HFCS- or sucrose-sweetened colas. Compared with the EIs under the no-beverage condition, the 3 colas and 1%-fat milk weakly suppressed EIs at lunch, whereas the diet cola did not. However, the effect was significant only for 1%-fat milk.

The present study used the conventional preload paradigm (25–27) and commercially available colas and 1%-fat milk. The statistical power was similar to that used in past research (9, 25). The study design was thus able to separate the effects of preload energy and preload volume. As had been noted in previous reports, diet cola suppressed hunger immediately after ingestion, but hunger ratings recovered sooner than did those after ingestion of caloric 1%-fat milk. Preload volume alone had no effect on EIs 120 min later. The amount of food consumed at lunch in the diet

TABLE 3

<table>
<thead>
<tr>
<th>Preload condition</th>
<th>Energy at lunch</th>
<th>Energy at lunch + preload</th>
<th>Volume of water at lunch</th>
<th>Weight of food at lunch without water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kcal</td>
<td>kcal</td>
<td>mL</td>
<td>g</td>
</tr>
<tr>
<td>HFCS 42</td>
<td>979 ± 40</td>
<td>1193 ± 40</td>
<td>415 ± 29</td>
<td>1004 ± 44</td>
</tr>
<tr>
<td>HFCS 55</td>
<td>969 ± 41</td>
<td>1182 ± 41</td>
<td>418 ± 27</td>
<td>1003 ± 39</td>
</tr>
<tr>
<td>Sucrose</td>
<td>957 ± 41</td>
<td>1170 ± 41</td>
<td>427 ± 31</td>
<td>1009 ± 44</td>
</tr>
<tr>
<td>Aspartame</td>
<td>1009 ± 39</td>
<td>1011 ± 39</td>
<td>437 ± 28</td>
<td>1033 ± 40</td>
</tr>
<tr>
<td>1%-fat Milk</td>
<td>916 ± 41</td>
<td>1129 ± 41</td>
<td>423 ± 26</td>
<td>961 ± 42</td>
</tr>
<tr>
<td>No beverage</td>
<td>1008 ± 40</td>
<td>1008 ± 41</td>
<td>522 ± 36</td>
<td>1125 ± 44</td>
</tr>
</tbody>
</table>

As the present study shows, the 3 cola beverages, which were sweetened with sucrose, HFCS 55, or HFCS 42, were perceived as equally sweet and significantly sweeter than diet cola. All 3 beverages showed identical temporal profiles of motivational ratings, which were different from the no-beverage condition. There were no differences between the reported temporal profiles for hunger, satiety, and the desire to eat obtained after the ingestion of HFCS- or sucrose-sweetened colas. Compared with the EIs under the no-beverage condition, the 3 colas and 1%-fat milk weakly suppressed EIs at lunch, whereas the diet cola did not. However, the effect was significant only for 1%-fat milk.

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TABLE 4

<table>
<thead>
<tr>
<th>Preload condition</th>
<th>Protein kcal</th>
<th>Carbohydrate kcal</th>
<th>Sugar kcal</th>
<th>Fat kcal</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFCS 42</td>
<td>165 ± 8</td>
<td>507 ± 21</td>
<td>181 ± 11</td>
<td>319 ± 17</td>
</tr>
<tr>
<td>HFCS 55</td>
<td>169 ± 9</td>
<td>503 ± 23</td>
<td>179 ± 10</td>
<td>310 ± 16</td>
</tr>
<tr>
<td>Sucrose</td>
<td>171 ± 9</td>
<td>482 ± 23</td>
<td>173 ± 12</td>
<td>315 ± 15</td>
</tr>
<tr>
<td>Aspartame</td>
<td>181 ± 9</td>
<td>504 ± 21</td>
<td>183 ± 10</td>
<td>338 ± 16</td>
</tr>
<tr>
<td>1%-fat Milk</td>
<td>156 ± 10</td>
<td>466 ± 22</td>
<td>161 ± 12</td>
<td>307 ± 15</td>
</tr>
<tr>
<td>No Beverage</td>
<td>166 ± 10</td>
<td>526 ± 21</td>
<td>195 ± 12</td>
<td>334 ± 15</td>
</tr>
</tbody>
</table>

As the present study shows, the 3 cola beverages, which were sweetened with sucrose, HFCS 55, or HFCS 42, were perceived as equally sweet and significantly sweeter than diet cola. All 3 beverages showed identical temporal profiles of motivational ratings, which were different from the no-beverage condition. There were no differences between the reported temporal profiles for hunger, satiety, and the desire to eat obtained after the ingestion of HFCS- or sucrose-sweetened colas. Compared with the EIs under the no-beverage condition, the 3 colas and 1%-fat milk weakly suppressed EIs at lunch, whereas the diet cola did not. However, the effect was significant only for 1%-fat milk.

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FIGURE 5. Timeline of sucrose inversion in the sucrose-containing cola used in this study. Each data point represents the mean of 3 samples taken from the same lot on the indicated date. As sucrose hydrolyzed over the weeks of the study, its concentration declined from 36% of total sugars to just above 10%. Free fructose increased from 32% to ≈45% of total sugar.
Although laboratory studies conducted with pure sugar solutions provide valuable data on fructose metabolism (12, 13), not all observations can be extrapolated to the human food supply. The hydrolysis of sucrose in soft drinks before consumption suggests that the substitution of HFCS for sucrose did not have the dramatic effects that had been claimed (2). The emerging view voiced in the scientific literature (33) and in the news media (34) is that any potential contribution of sugars to obesity is unlikely to be mediated by metabolic effects that are unique to HFCS.

We thank Susan L. Adams for assistance in the planning of the test lunch, Robyn Sakamoto for assistance in conducting the study, and Chris Prall for VAS software development. The Nutritional Sciences Program has received prior research support from industry sources in the United States and the European Union.

The authors’ responsibilities were as follows—PM, MMP, and AD: study design and writing of the manuscript; PM and MMP: study implementation and data collection; PM, MMP, and AD: statistical analysis and revision of the dietary data. None of the authors had a personal or financial conflict of interest.

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Extract of *Salacia oblonga* lowers acute glycemia in patients with type 2 diabetes\textsuperscript{1–4}

Jennifer A Williams, Yong S Choe, Michael J Noss, Carl J Baumgartner, and Vikkie A Mustad

**ABSTRACT**

**Background:** Two previous studies tested the efficacy of *Salacia oblonga* extract in healthy adults.

**Objective:** This study evaluated the effect of an herbal extract of *Salacia oblonga* on postprandial glycemia and insulinemia in patients with type 2 diabetes after ingestion of a high-carbohydrate meal.

**Design:** Sixty-six patients with diabetes were studied in this randomized, double-blinded crossover study. In a fasted state, subjects consumed 1 of the following 3 meals: a standard liquid control meal, a control meal + 240 mg *Salacia oblonga* extract, and a control meal + 480 mg *Salacia oblonga* extract. Serum glucose and insulin samples were measured at baseline and at postprandial intervals up to 180 min.

**Results:** Both doses of the *Salacia* extract significantly lowered the postprandial positive area under the glucose curve (14% for the 240 mg extract and 22% for the 480 mg extract) and the adjusted peak glucose response (19% for the lower dose and 27% for the higher dose of extract) to the control meal. In addition, both doses of the herbal extract significantly decreased the postprandial insulin response, lowering both the positive area under the insulin curve and the adjusted peak insulin response (14% and 9%, respectively, for the 240 mg extract; 19% and 12%, respectively, for the 480 mg extract) in comparison with the control meal.

**Conclusions:** The extract of *Salacia oblonga* lowers acute glycemia and insulinemia in persons with type 2 diabetes after a high-carbohydrate meal. The results from this study suggest that *Salacia* may be beneficial to this population for postprandial glucose control. *Am J Clin Nutr* 2007;86:124–30.

**KEY WORDS** *Salacia oblonga*, postprandial glycemia, herbal extract, α-glucosidase inhibitor

**INTRODUCTION**

Diabetes results in both public health and financial burdens to the society. In 2005, the estimated total prevalence of diabetes in the United States was 20.8 million persons, or 7.0% of the population (1). In addition, 20.9% of persons aged ≥60 y has the disease, which was the sixth leading cause of death in 2002 (1). The financial burden of this disease translates to an estimated $132 billion in medical expenditures, disability, and lost productivity in the United States (2). Because the risk of developing long-term complications can be dramatically reduced with appropriate glycemic control (3), food ingredients that can attenuate postprandial glucose in persons with diabetes would be useful.

One such ingredient, the root extract of *Salacia oblonga*, inhibits the breakdown of oligosaccharides and polysaccharides into monosaccharides by competitive inhibition of α-glucosidase activity within the lumen of the intestinal tract. Historically, the *Salacia* plant has been used as part of the traditional Ayurvedic system of Indian medicine to treat diseases such as diabetes (4); currently, extracts of *Salacia* are consumed in commercial foods and food supplements in Japan for the treatment of diabetes and obesity. Yoshikawa et al (5) isolated active components of a *Salacia* extract and concluded that their mode of action was the inhibition of α-glucosidase enzymes. Two compounds isolated from *Salacia* extracts, salacinol and kotalanol, inhibit the action of the small intestinal enzymes sucrase, maltase, and isomaltase (6). Therefore, naturally derived α-glucosidase inhibitors may be powerful nutritional adjuncts for the treatment of diabetes mellitus by lowering postprandial blood glucose.

The potential genotoxicity and safety of *Salacia oblonga* extract (SOE) were evaluated with a standard battery of tests (reverse mutation assay, chromosomal aberrations assay, mouse micronucleus assay) recommended by the US Food and Drug Administration for food ingredients (7), and in a 92–93-d feeding study in rats with doses of 250, 1250, and 2500 mg · kg\(^{-1}\) · d\(^{-1}\) by oral gavage (8). SOE was determined not to be genotoxic, and the no observable adverse effect level was determined to be 2500 mg · kg\(^{-1}\) · d\(^{-1}\) after daily subchronic oral gavage administrations to rats (8).

Two previous studies in healthy adults were conducted to determine the effect of SOE on postprandial glycemia and insulinemia after a control meal (9, 10). Both studies showed efficacy on postprandial glycemia with 1000 mg SOE. The primary objective of this study was to compare the effect of 2 different doses of SOE on the glycemic and insulinemic response in patients with type 2 diabetes. Because previous experiments were conducted with healthy subjects, a logical next step was to test SOE within...
the population most likely to benefit from its effects on postprandial glycemia—patients with type 2 diabetes.

SUBJECTS AND METHODS

Subjects

This was a 2-center, randomized, double-blinded, 3-period, 3-treatment crossover study. Eighty-two subjects were enrolled, 41 per site, and 66 successfully completed the study according to protocol by meeting all entry criteria, complying with meal tolerance test preparation, having nonmissing data for the primary variable at all 3 treatment visits, and consuming all test products. For the 16 subjects who were not included in the protocol evaluable analysis, 8 of the subjects had missing or mistimed blood samples, 2 were ineligible, 2 had changes to their antihyperglycemic medications during the study, 1 withdrew before treatment visits, 1 was randomly assigned incorrectly, 1 withdrew after a visit because of the subject’s complaint of shakiness, and 1 withdrew at a treatment visit after symptoms of nausea and vomiting. The 66 protocol evaluable subjects (53 men and 13 nonpregnant, nonlactating women) had the following characteristics: mean age (±SEM) of 61.3 ± 1.1 y, weight of 87.3 ± 1.5 kg, and body mass index (BMI; in kg/m²) of 28.8 ± 0.4. The self-reported ethnicity of the subjects was 1 American Indian or Alaskan native, 7 African Americans, and 58 non-Hispanic whites. All subjects were patients with type 2 diabetes mellitus based on use of antihyperglycemic medication(s), but they did not use exogenous insulin for glucose control. These subjects were free from hepatic disease, active malignancy, end-stage organ failure, chronic infectious disease, or active metabolic disease (excluding diabetes) that would interfere with nutrient absorption, metabolism, or excretion. In addition, subjects did not have a recent infection, surgery, or corticosteroid treatment and did not have a significant cardiovascular event within 12 wk of study start. The study protocol was reviewed and approved by the Schulman Associates Institutional Review Board (Cincinnati, OH), and all enrolled subjects provided informed consent before the start of the study. The trial was conducted in accordance with the principles of Good Clinical Practice and the Declaration of Helsinki.

Study procedures

The 3 meal tolerance tests were administered in random order at least 3 d apart at each of the 2 clinical research centers. The 3 treatments were as follows: 1) control was 510 g (two 8-fl oz bottles) of a liquid meal replacement (chocolate Ensure; Ross Products Division, Abbott Laboratories, Columbus, OH) and 30.0 g maltodextrin (Polycose; Ross Products Division, Abbott Laboratories), 2) control + 240 mg SOE (agglomerated with 960 g maltodextrin), and 3) control + 480 mg SOE (agglomerated with 1920 g maltodextrin). The manufacture release criteria for the SOE lot 040304 was 46 μg/mL (IC₅₀) inhibition of α-glucosidase action. The SOE ingredient is a proprietary ethanol and water extract produced at Takama Co Ltd (Yamaguchi, Japan), supplied through Tanabe Seiyaku Co, Ltd (Osaka, Japan). The control meal consisted of 110 g carbohydrate (55 g maltodextrin, 31 g sucrose, 25 g corn syrup), 12 g fat, 18 g protein, and 620 kcal. An unblinded product coordinator opened sealed envelopes containing the randomization sequences for subjects and prepared the test meals according to the assigned order. The test meals were prepared by mixing with a blender.

The product coordinator was independent from all other study procedures. Subjects prepared for each meal tolerance test by consuming an average of ≥150 g carbohydrate/d for the 3 d before test visit, which was documented on a 3-d diet record. Subjects did not consume alcohol or participate in strenuous exercise for the 24 h before the test visits. Subjects fasted (except for water and antihyperglycemic medications) for 8–16 h before the meal tolerance tests, and subjects refrained from taking oral antihyperglycemic medications on the morning of test visits. Subjects consumed the test meals within 10 min, and all postprandial blood samples were drawn based on the time of start of meal consumption, which was considered time zero. Eight venous blood samples were drawn for glucose and insulin measurements at a central laboratory (Quest Diagnostics, Collegeville, PA) at the following times: baseline (before meal consumption) and 30, 45, 60, 90, 120, 150, and 180 min after the start of meal consumption. The serum samples were allowed to clot in serum separator tubes at room temperature and centrifuged at 1000 × g for 15 min at room temperature. Glucose was measured with the use of an enzymatic method (hexokinase glucose), and insulin was measured with the use of a radioimmunoassay procedure (DPC Immulite 2000 assay; DPC Biermann, Bad Nauheim, Germany).

Statistics

This was a randomized, double-blinded, 3-period, 3-treatment, crossover study conducted at 2 sites. A total of 82 subjects were randomly assigned to 1 of 6 treatment sequences, 1) A-B-C, 2) B-C-A, 3) C-A-B, 4) C-B-A, 5) A-C-B, and 6) B-A-C (A, control; B, control + 240 mg SOE; C, control + 480 mg SOE). Sixty-six subjects were included in the protocol evaluable analysis, which was the minimum number of subjects required in the power analysis before study start.

With the use of data from a previous study, a sample size of 66 was calculated to have 83% power to detect a 20% of the control difference in treatment means with the use of a single-group repeated measures analysis of variance with a 0.05 significance level. Statistical software NQUERY ADVISOR 5.0 (Statistical Solutions, Los Angeles, CA) was used for the sample size estimation.

Each variable was analyzed by using parametric or nonparametric (if declared nonnormal) 3-period, 3-treatment, crossover analysis. The parametric analysis was performed by using repeated measures analysis of variance with variance components covariance structure and Satterthwaite df with site, treatment, and period as fixed effects and subject nested within site as random effect. The 3 pairwise differences of least squares means of the treatments were tested with the use of Tukey-Kramer P value adjustments. If the parametric approach was determined to be inappropriate by the Shapiro-Wilk test for normality, then 3 pairwise treatment differences were analyzed with the use of signed rank test with stepdown Bonferroni (Holm) P value adjustments. A result was declared to be statistically significant if and only if a P value of an analysis was < 0.05. Statistical software SAS release 8.2 (SAS Institute Inc, Cary, NC) was used for the analyses.

RESULTS

Serum glucose

No significant differences were observed between treatments for baseline serum glucose concentrations. Baseline and postprandial values for both serum glucose and insulin are presented.
TABLE 1
Serum glucose and insulin by treatment in all available subjects

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Control</th>
<th>Control + 240 mg SOE</th>
<th>Control + 480 mg SOE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline fasting (mg/dL)</td>
<td>143 ± 5</td>
<td>144 ± 5</td>
<td>145 ± 5</td>
</tr>
<tr>
<td>Peak value (mg/dL)</td>
<td>303 ± 9</td>
<td>272 ± 7</td>
<td>263 ± 7</td>
</tr>
<tr>
<td>Adjusted peak value (mg/dL)</td>
<td>158 ± 7</td>
<td>130 ± 5</td>
<td>116 ± 5</td>
</tr>
<tr>
<td>AUC (mg·min/dL)</td>
<td>44 727 ± 1339</td>
<td>41 742 ± 1196</td>
<td>40 570 ± 1145</td>
</tr>
<tr>
<td>Positive AUC (mg·min/dL)</td>
<td>18 570 ± 860</td>
<td>16 100 ± 732</td>
<td>14 328 ± 726</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Insulin</th>
<th>Control</th>
<th>Control + 240 mg SOE</th>
<th>Control + 480 mg SOE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline fasting (µIU/mL)</td>
<td>14 ± 1</td>
<td>14 ± 1</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Peak value (µIU/mL)</td>
<td>80 ± 6</td>
<td>73 ± 5</td>
<td>72 ± 5</td>
</tr>
<tr>
<td>Adjusted peak value (µIU/mL)</td>
<td>67 ± 5</td>
<td>60 ± 4</td>
<td>58 ± 5</td>
</tr>
<tr>
<td>AUC (µIU·min/mL)</td>
<td>10 693 ± 997</td>
<td>9 552 ± 751</td>
<td>9 099 ± 630</td>
</tr>
<tr>
<td>Positive AUC (µIU·min/mL)</td>
<td>8 223 ± 846</td>
<td>7 122 ± 598</td>
<td>6 520 ± 525</td>
</tr>
</tbody>
</table>

1 All values are x ± SEM; n = 61–79 (sample size differed according to data available for specific variables). SOE, Salacia oblonga extract; AUC, area under the curve. Values in a row with different superscript letters are significantly different, P < 0.05.

in Table 1 and Table 2 for all available subjects and the protocol evaluable set of subjects, respectively. Because the protocol evaluable analysis was the primary analysis and because results for both datasets are similar, only protocol evaluable data are presented in the data figures. The net changes in serum glucose concentrations are shown in Figure 1. Both doses of SOE significantly decreased the adjusted peak serum glucose response, 19% and 27%, respectively, for the 240-mg and 480-mg doses of SOE compared with the control meal alone (Figure 2). In addition, both doses of SOE significantly lowered the positive serum glucose area under the curve (AUC) for 0–180 min postprandial in comparison with the control meal, with a 14% reduction for the 240-mg dose and a 22% decrease for the 480-mg dose (Figure 3).

**Serum insulin**

No significant differences were observed in serum insulin across treatments at baseline. The net changes in serum insulin concentrations are shown in Figure 4. The 2 doses of SOE significantly lowered the adjusted peak serum insulin response 9% and 12%, respectively, for the 240-mg and 480-mg doses of SOE in comparison to the response by the control meal alone (Figure 5). Also, compared with the control meal, both doses of SOE significantly reduced the postprandial positive serum insulin AUC for 0–180 min, with a 14% decrease for the 240-mg dose and a 19% decrease for the 480-mg dose (Figure 6).

**Safety and gastrointestinal tract tolerance**

Twenty-three protocol evaluable subjects experienced adverse events during the study with all but one (moderate) event considered mild in severity. Sixteen of these subjects had symptoms related to gastrointestinal (GI) tolerance such as flatulence, belching, abdominal pain, nausea, and diarrhea which lasted ≤24 h after a test meal. Two subjects had GI symptoms after ingesting the control meal, 8 did after the 240-mg dose of SOE, and 12 subjects experienced GI symptoms after ingesting the 480-mg dose of SOE. Ten subjects had mild adverse events not related to GI symptoms, which included flu or sinus symptoms, bruised arms as a result of blood draws, study product aftertaste, vertigo, and pain or injury to the back or extremities.

**DISCUSSION**

This study presents the first published results on the effects of SOE on postprandial blood glucose in patients with diabetes.
Both doses of the herbal extract had significant effects on postprandial glycemia and insulinemia after a high-carbohydrate meal. The results from the current study corroborate with previous data observed in healthy adult subjects (9, 10). However, the doses of extract for this study are much lower than the 1000-mg dose found to be efficacious in the 2 previous experiments. One possible reason for the difference is that a different extraction process was used for product in the current trial. The extract used in the 2 trials with healthy subjects was made by a hot water extraction process, and the SOE for the current study was created by an ethanol and water extraction, which may result in greater concentrations of active components. It is also possible that the glycemic responses of patients with diabetes were different from that of the healthy subjects of the 2 previous studies by evidence of the prolonged glucose excursion, resulting in larger differences between treatments for positive AUC. In addition, the difference in efficacious doses of SOE may be accounted for by the different amount of carbohydrate in the 2 control meals: 82 g carbohydrate (61% of energy intake) in the healthy subject study compared with 110 g carbohydrate (71% of energy intake) in the present study with patients with type 2 diabetes.

Postprandial glycemia is important for overall glycemic control. An expert panel concluded that postprandial hyperglycemia is a risk indicator for microvascular and macrovascular complications in both patients with type 2 diabetes and persons with impaired glucose tolerance (11). In a number of prospective clinical trials, postprandial glucose excursions were linked to increased mortality from cardiovascular disease, and decreasing

FIGURE 1. Mean (±SEM) net change from baseline in serum glucose concentrations in patients with type 2 diabetes in the 3 meal groups (n = 62–66). SOE, Salacia oblonga extract. No statistical analyses were conducted for these variables.

FIGURE 2. Mean (±SEM) adjusted peak serum glucose concentrations in patients with type 2 diabetes after the control meal and after the control meal plus 2 different doses of Salacia oblonga extract (SOE): 160 ± 7 mg/dL (n = 55) for the control meal, 130 ± 6 mg/dL (n = 63) for the control plus 240 mg SOE meal, and 116 ± 6 mg/dL (n = 63) for the control plus 480 mg meal. Values with different superscript letters are significantly different. Both doses of SOE significantly reduced the adjusted peak value compared with the control meal (P < 0.0001 for both), and the 480-mg dose significantly reduced the adjusted peak value compared with the 240-mg dose of SOE (P = 0.0050). The data were analyzed with the use of a mixed-model repeated-measures ANOVA with variance components covariance structure and Satterthwaite df with site, treatment, and visit as fixed effects and subject nested within site as a random effect. The 3 pairwise differences of least-squares means of the treatments were tested with the use of Tukey-Kramer P value adjustments.
postprandial glucose by therapeutic agents will decrease the progression of retinopathy, neuropathy, and nephropathy in patients with diabetes (12). So, when designing strategies for reducing the burden of diabetic complications, both a quantitative effect of hyperglycemia (postprandial hyperglycemia and glycated hemoglobin) and a qualitative component (glucose stability throughout the day) should be considered (13).

Therapy with α-glucosidase inhibitors can benefit patients with diabetes beyond lowering postprandial glucose. For example, in the STOP-NIDDM (Study to Prevent NIDDM) trial, the group randomly assigned to acarbose not only had a reduction in body weight, BMI, waist and hip circumferences, systolic and diastolic blood pressures, blood triacylglycerols, and 2-h postprandial glucose during a 3-y period following subjects with impaired glucose tolerance but also experienced a significantly reduced incidence of cardiovascular events and hypertension (14). A meta-analysis of 7 long-term studies showed that α-glucosidase inhibitors significantly reduce the risk of myocardial infarction or any cardiovascular event (15). Recent research testing SOE shows that the extract improves cardiac lipid metabolism and postprandial hyperlipidemia in rats and possesses activating properties for peroxisome-proliferator activated receptor-α (16, 17). In addition, a study conducted in rats shows that *Salacia reticulata*, another species of the *Salacia* plant,
inhibits lipase and has a mild antiobesity effect (18). Therefore, the application for \(H\text{9251}\)-glucosidase inhibitors in patients with type 2 diabetes may extend beyond postprandial glucose control.

Of 66 subjects, only 24% had GI symptoms because of study product consumption, with 12% of all subjects having symptoms with the 240-mg dose of SOE and 18% with the 480-mg dose. It is not uncommon for \(H\text{9251}\)-glucosidase inhibitors to cause GI symptoms because of fermentation of undigested carbohydrates in the bowel (19). The difference in the number of GI events between the 2 doses of SOE is obvious and probably dose dependent. The \(H\text{9251}\)-glucosidase inhibitor acarbose was found to cause a dose-dependent increase in the amount of carbohydrate entering the colon, which leads to an increase in colonic fermentation and GI symptoms (20). In addition, the large amount of carbohydrate in the control meal (110 g) for this study may have caused some of the intolerance. Daily carbohydrate consumption for persons

**FIGURE 5.** Mean (±SEM) adjusted peak serum insulin concentrations in patients with type 2 diabetes after the control meal and after the control meal plus 2 different doses of Salacia oblonga extract (SOE): 65 ± 5 \(\mu\)IU/mL \((n = 55)\) for the control meal, 59 ± 5 \(\mu\)IU/mL \((n = 63)\) for the control + 240 mg SOE meal, and 57 ± 5 \(\mu\)IU/mL \((n = 61)\) for the control + 480 mg SOE meal. Values with different superscript letters are significantly different. Both the 240-mg \((n = 53)\) and 480-mg \((n = 52)\) doses of SOE significantly reduced the adjusted peak value compared with the control meal \((P = 0.0126 \text{ and } P = 0.0021, \text{ respectively})\), but the values after the 2 doses of the extract did not differ significantly. The 3 pairwise treatment differences were analyzed with the use of a signed-rank test with stepdown Bonferroni (Holm) \(P\) value adjustments.

**FIGURE 6.** Mean (±SEM) positive area under the curve (AUC) for serum insulin concentration in patients with type 2 diabetes from 0 to 180 min after the control meal and after the control meal plus 2 different doses of Salacia oblonga extract (SOE): 8092 ± 889 \(\mu\)IU·min/mL \((n = 65)\) for the control meal, 6986 ± 651 \(\mu\)IU·min/mL \((n = 66)\) for the control + 240 mg SOE meal, and 6551 ± 601 \(\mu\)IU·min/mL \((n = 66)\) for the control + 480 mg SOE meal. Values with different superscript letters are significantly different. Both the 240-mg and 480-mg doses of SOE significantly reduced the positive AUC compared with the control meal \((P = 0.0183 \text{ and } P = 0.0004, \text{ respectively})\), but the values after the 2 doses of the extract did not differ significantly. The 3 pairwise treatment differences were analyzed with the use of a signed-rank test with stepdown Bonferroni (Holm) \(P\) value adjustments.
with diabetes is typically <50% of energy intake (20), so the large quantity of carbohydrate, 71% of test meal calories, may have increased symptoms in study subjects. In addition to lowering the amount of carbohydrates consumed, another way to decrease GI symptoms would be to adjust the dose of SOE over a period of time, which is a common practice when administering α-glucosidase inhibitors (21).

Novel ingredients such as SOE may be ideal for medical nutritional therapy. Lifestyle modifications consisting of diet and exercise were shown to be effective for reducing macrovascular complications in patients with type 2 diabetes and for lowering relative risk of developing the disease in high-risk persons (22, 23). Although diabetes and its encompassing symptoms can be altered by diet and exercise, behavioral obstacles can prevent the occurrence of appropriate changes. Several situational obstacles for adults with diabetes were identified for dietary adherence, including ingredients that lower postprandial glycemia, such as resisting temptation, eating out, feeling deprived, planning meals, social events, and so forth (24, 25). Nutritional adjuncts, including ingredients that lower postprandial glycemia, can provide more flexibility with meal planning and eating with family and friends. These adjuncts may also enable patients with type 2 diabetes to eat a greater variety of foods and carbohydrates with less restriction.

Historical and present uses of Salacia in India and Japan show that this herbal extract is used as a nutritional adjunct, either as a tea or supplement, taken with meals for its antidiabetic properties. SOE does lower postprandial glycemia in patients with type 2 diabetes. So, the long-term benefits of this herbal extract on glycemic control should be explored within this population to determine its value in the realm of nutritive therapy.

We thank the study subjects, study coordinators Pegi Deuss and Jane Gillingham, and numerous other study personnel for their dedication and invaluable contributions.

The author’s responsibilities were as follows—JAW: study design, review of the original data, and draft of manuscript; YSC: statistical design, final analysis of the data, and manuscript preparation; MJN and CJB (principal investigators): conduct of study at their clinical sites; and VAM: oversight of the study and editorial revisions of the manuscript. All authors reviewed and approved the final version of manuscript. JAW, YSC, and VAM have personal and financial conflicts of interest as employees of the sponsor company, Abbott Laboratories. MJN and CJB do not have personal or financial conflicts of interest.

REFERENCES
Plasma vitamin B-6 forms and their relation to transsulfuration metabolites in a large, population-based study

Øivind Midttun, Steinar Hustad, Jørn Schneede, Stein E Vollset, and Per M Ueland

ABSTRACT
Background: Vitamin B-6 exists in different forms; one of those forms, pyridoxal 5′-phosphate (PLP), serves a cofactor in many enzyme reactions, including the transsulfuration pathway, in which homocysteine is converted to cystathionine and then to cysteine. Data on the relations between indexes of vitamin B-6 status and transsulfuration metabolites in plasma are sparse and conflicting.

Objective: We investigated the distribution and associations of various vitamin B-6 species in plasma and their relation to plasma concentrations of transsulfuration metabolites.

Design: Nonfasting blood samples from 10,601 healthy subjects with a mean age of 56.4 y were analyzed for all known vitamin B-6 vitamers, folate, cobalamin, riboflavin, total homocysteine, cystathionine, total cysteine, methionine, and creatinine. All subjects were genotyped for the methylenetetrahydrofolate reductase (MTHFR) 677C→T polymorphism.

Results: Plasma concentrations of the main vitamin B-6 vitamers—PLP, pyridoxal, and 4-pyridoxic acid—were strongly correlated. Among the vitamin B-6 vitamers, PLP showed the strongest and most consistent inverse relation to total homocysteine and cystathionine, but the dose response was different for the 2 metabolites. The PLP–total homocysteine relation was significant only in the lowest quartile of the vitamin B-6 distribution and was strongest in subjects with the MTHFR 677TT genotype, whereas cystathionine showed a graded response throughout the range of vitamin B-6 vitamer concentrations, and the effect was not modified by the MTHFR 677C→T genotype.

Conclusion: This large population-based study provided precise estimates of the relation between plasma concentrations of vitamin B-6 forms and transsulfuration metabolites as modified by the MTHFR 677C→T genotype.

INTRODUCTION
Vitamin B-6 is a versatile enzyme cofactor that is involved in ≈100 enzymatic reactions (1). Vitamin B-6 exists in 7 forms: pyridoxine, pyridoxine 5′-phosphate (PNP), pyridoxal, pyridoxal 5′-phosphate (PLP), pyridoxamine, pyridoxamine 5′-phosphate (PMP), and the catabolite 4-pyridoxic acid (PA). Pyridoxal and PLP are the major vitamin B-6 forms obtained from animal food products, whereas pyridoxine, pyridoxamine, PNP, and PMP are the main forms obtained from plants (1). Pyridoxine is also the form given as vitamin B-6 supplement. Vitamin B-6 is absorbed in the jejunum and metabolized in the liver (2), which releases PLP (3) with pyridoxal and PA (2) into the circulation. The major catabolic pathway in humans is the hydrolysis of the metabolically active form PLP to pyridoxal, which is followed by oxidation to PA (4).

Orally supplemented pyridoxine is absorbed quickly, which results in a plasma pyridoxine peak that disappears in a few hours (2, 5, 6), strong increases in plasma pyridoxal (2, 5, 7, 8) and PA (2, 5, 7) that normalize in several hours, and an increase in plasma PLP that lasts >24 h (2, 5, 7–9). PLP, pyridoxal, and PA are the major vitamin B-6 forms in plasma (10–12), where most PLP (3), and some pyridoxal—but no PA or pyridoxine—are protein-bound (13). Free plasma pyridoxal but not protein-bound PLP can cross cell membranes (3, 14, 15). Once inside the cell, pyridoxal may be converted to PLP, which is the metabolically active form (1). Plasma PLP is the most commonly used vitamin B-6 index (14, 16, 17). However, pyridoxal (14, 18, 19) and the combinations PLP plus pyridoxal (14, 20) and PLP plus PA (21–23) have also been suggested as useful markers of vitamin B-6 status.

PLP serves as cofactor in both steps in the transsulfuration pathway, in which cystathionine β-synthase and cystathionine γ-lyase convert homocysteine to cystathionine and then to cysteine (24). An inverse relation between plasma PLP and total homocysteine (tHcy) in nonfasting (25) and fasting (23, 26) subjects has been reported by some authors, but most found no such relation (27–35). Similarly, some studies reported a tHcy-lowering effect of pyridoxine supplementation (36, 37), but most investigators found no such effect in fasting (29, 33, 38–45) or nonfasting (46) subjects. An inverse relation between plasma cystathionine and PLP during fasting was reported (35), and both

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fasting (32) and nonfasting (46) plasma cystathionine concentrations were reduced by pyridoxine supplementation. Studies of the relation of plasma PLP (23, 35, 47) and pyridoxine supplementation (32, 43) to total cysteine (tCys) has been negative.

The enzyme methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) catalyzes the irreversible conversion of 5,10–methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as methyl donor in the remethylation of homocysteine to methionine. Homozygosity for the common MTHFR 677C→T polymorphism is associated with higher plasma tHcy concentrations (48) and stronger inverse relations of plasma tHcy with folate, vitamin B-12, riboflavin (48), and possibly also with PLP (49).

This report focuses on the concentrations and interrelations of all forms of vitamin B-6 and on the relations of the major vitamin B-6 forms with plasma tHcy and its transsulfuration metabolites cystathionine and cysteine. We also investigated the possible effect modification by the MTHFR 677C→T genotype. The study was carried out in a large cohort of healthy Norwegian subjects.

SUBJECTS AND METHODS

Subjects and recruitment

The present study includes 10 601 healthy subjects from the Norwegian Colorectal Cancer Prevention cohort (50) who were randomly selected from the population registries of Oslo and Telemark counties in Norway from 1999 through 2001. All of the participants (men and women) were 50–64 y old.

Written informed consent was obtained from all participants. The Regional Ethics Committee and The Data Inspectorate approved the study protocol.

Blood collection and biochemical analyses

Blood was drawn from nonfasting subjects during normal working hours at 3 study centers: Ullevål Hospital in Oslo, Telemark Hospital in Skien, and Rjukan Hospital in Rjukan (all: Norway). The blood was centrifuged at 1100 × g for 10 min at 23 °C, and serum and plasma (which had been drawn into tubes containing EDTA) were separated and kept at −80 °C until they were analyzed.

Plasma tHcy, tCys (51), vitamin B-6 and riboflavin (12), serum folate (52), cobalamin (53) concentrations were measured, and MTHFR 677C→T genotypes (54) were determined according to published methods. Cystathionine was analyzed by including it and a deuterated internal standard (d4-cystathionine) in an existing liquid chromatography–tandem mass spectrometry assay (12). Ion pairs were 222.9/133.9 for cystathionine and 226.9/137.9 for d4-cystathionine. Creatinine and total methionine (sum of methionine and methionine sulfoxide) were analyzed by including them and their deuterated internal standards (d3-creatine and d4-methionine) in a liquid chromatography–tandem mass spectrometry assay (55) by using the ion pairs 114/44.2, 150.2/104, 166.1/73.9, 117/47.2, and 154/108, respectively.

Statistical analysis

Concentrations are given as means and medians (5th, 95th percentiles). Concentration means, age, and sex across genotypes were compared by linear regression after adjustment (where appropriate) for age, sex, and study center. Relations between the vitamin B-6 vitamers PLP, pyridoxal, and PA; other B vitamins; and metabolites were investigated by using partial Spearman correlation after adjustment for age, sex, and study center. The relations between PLP, pyridoxal, and PA were also presented as scatterplots with lowess regression curves (56) with the smoother span and delta both set at 0.01.

The relations between the metabolites and various forms of vitamin B-6 were assessed in multiple linear regression models. Separate regression models were constructed for each of the major vitamin B-6 forms. Age was included as a continuous variable. Categorical variables indicating study center enrollment were used. Vitamin B-6 forms, creatinine, folate, cobalamin, riboflavin, and methionine were included as indicator variables, with one variable used for each concentration quartile. The regression coefficients estimated the difference in mean tHcy between the chosen reference category and the other categories. Mean metabolite concentrations across quartiles of PLP, pyridoxal, or PA were also tested for linear trend. We investigated the possible interaction between the MTHFR 677C→T genotype and a vitamin B-6 vitamer by including product terms between genotype and the vitamer concentration in multiple linear regression models in which the transsulfuration metabolites served as the dependent variable; all primary variables were retained in the model. Tests were 2-tailed, and P < 0.05 was considered significant.

Statistical analyses were performed by using SPSS software (version 11.0; SPSS, Chicago, IL), except for the lowess regression, which was computed by using R (57).

RESULTS

Population characteristics

The study population (n = 10 601, 49.2% male) was predominantly (>98%) white and had a mean age of 56.4 y (Table 1). MTHFR 677C→T genotype frequencies were 51.4%, 40.6%, and 8.0% for the CC, CT, and TT genotypes, respectively, and neither sex nor age varied between the genotypes (Table 1).

Vitamin B-6 vitamers

PLP, pyridoxal, and PA were present in all plasma samples. The concentrations and distribution of these vitamers are summarized in Table 1 and Figure 1. Median (5th, 95th percentiles) concentrations were 43.7 (16–139), 9.5 (5–39), and 20.3 (10–100) nmol/L for PLP, pyridoxal, and PA, respectively. Only PLP was related to the MTHFR 677C→T polymorphism, and its lowest concentrations (as were those of folate) were found in subjects with the TT genotype (Table 1). The concentration of PLP ranged from 4 to 1100 nmol/L, whereas pyridoxal and PA had a wider concentration range of 1 to 4500 nmol/L. All 3 species showed a skewed distribution with a long tail in the upper region, and the distributions became essentially symmetric after log transformation (Figure 1).

Pyridoxine and pyridoxamine were detected in 1.9% and 0.85% of the samples; their maximum concentrations were 2970 and 465 nmol/L, respectively. PMP and PNP were rarely detected in plasma; if they were present, their concentrations were always close to the lower limit of quantification of the assay (ie, 0.2 nmol/L for PNP and 4 nmol/L for PMP).

The concentrations of the main species—PLP, pyridoxal, and PA—were strongly related (Figure 1). The plots of PLP versus pyridoxal or PA showed the steepest increase at higher PLP concentrations, whereas pyridoxal and PA had a linear relation
throughout the range of concentrations. All correlations were highly significant \( (P < 0.001) \) but were somewhat stronger between PLP and pyridoxal (Spearman \( r = 0.80 \)) and between pyridoxal and PA (\( r = 0.79 \)) than between PLP and PA (\( r = 0.67 \)) (Table 2).

The vitamin B-6 vitamers showed moderate correlations with folate and riboflavin (\( r = 0.35–0.45 \)) and a weaker correlation with cobalamin (\( r = 0.14–0.18 \)). Methionine and creatinine were more strongly associated with PLP and PA, respectively, than with the other vitamin B-6 vitamers (Table 2).

### Homocysteine

The median (5th, 95th percentiles) tHcy concentration for all subjects combined was 10.2 (6.8–16.4) \( \mu \text{mol/L} \), and the concentration increased with the number of MTHFR 677T alleles \( (P < 0.001; \text{Table 1}) \). Plasma tHcy was negatively related to folate, cobalamin, and riboflavin and positively related to creatinine (Table 2).

The association of plasma tHcy with either PLP, pyridoxal, or PA was assessed by using multiple regression analyses after

---

**TABLE 1**

Characteristics of the study population

<table>
<thead>
<tr>
<th>All subjects ((n = 10,601))</th>
<th>MTHFR 677C→T genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Mean} ) Median Percentiles(^3 )</td>
<td>( \text{Mean} ) Median Percentiles(^3 )</td>
</tr>
<tr>
<td><strong>Total homocysteine</strong> ((\mu \text{mol/L}))</td>
<td>10.8 10.2 (6.8, 16.4)</td>
</tr>
<tr>
<td><strong>Cystathionine</strong> ((\mu \text{mol/L}))</td>
<td>0.237 0.190 (0.091, 0.525)</td>
</tr>
<tr>
<td><strong>Total cysteine</strong> ((\mu \text{mol/L}))</td>
<td>285.3 283.7 (237.1, 338.2)</td>
</tr>
<tr>
<td><strong>PLP</strong> ((\text{nmol/L}))</td>
<td>62.7 48.0 (18.7, 152.4)</td>
</tr>
<tr>
<td><strong>PL</strong> ((\text{nmol/L}))</td>
<td>21.5 10.0 (5.2, 41.1)</td>
</tr>
<tr>
<td><strong>PA</strong> ((\text{nmol/L}))</td>
<td>42.0 20.4 (10.3, 110.0)</td>
</tr>
<tr>
<td><strong>Folate</strong> ((\text{nmol/L}))</td>
<td>17.1 13.7 (6.6, 39.4)</td>
</tr>
<tr>
<td><strong>Cobalamin</strong> ((\text{pmol/L}))</td>
<td>332.0 307.2 (172.0, 535.9)</td>
</tr>
<tr>
<td><strong>Riboflavin</strong> ((\text{nmol/L}))</td>
<td>18.1 10.5 (4.1, 55.9)</td>
</tr>
<tr>
<td><strong>Total methionine</strong> ((\text{nmol/L}))</td>
<td>23.5 22.5 (16.2, 34.9)</td>
</tr>
<tr>
<td><strong>Creatine</strong> ((\mu \text{mol/L}))</td>
<td>69.9 68.9 (50.6, 92.2)</td>
</tr>
<tr>
<td><strong>Age (y)</strong></td>
<td>56.4 55 (51, 63)</td>
</tr>
<tr>
<td><strong>Male (%)</strong></td>
<td>49.2 48.2</td>
</tr>
</tbody>
</table>

\(^1\) All concentrations are in plasma, except folate, which is in serum. MTHFR, methylenetetrahydrofolate reductase; PLP, pyridoxal 5’-phosphate; PL, pyridoxal; PA, pyridoxic acid.

\(^2\) Means across genotypes were modelled by linear regression and adjusted (where appropriate) for age, sex, and study center.

\(^3\) 5th, 95th percentiles.
adjustment for other B vitamins, creatinine, study center, age, and sex (Table 3). Plasma tHcy increased with decreasing concentrations of PLP only in the lowest PLP quartile. Furthermore, the tHcy differences across quartiles were investigated separately in the MTHFR 677C→T genotypes and was most pronounced (2.18 μmol/L) in the TT genotype (P < 0.001 for interaction between PLP and MTHFR). Plasma tHcy was similarly related to pyridoxal and PA, but the associations were in general weaker than those with pyridoxal (Table 3).

Cystathionine and cysteine

The median (5th, 95th percentiles) concentrations for all subjects combined were 0.190 (0.091–0.525) μmol/L for cystathionine and 283.7 (237–338.2) μmol/L for tCys; the concentration of cystathionine did not vary with MTHFR 677C→T genotype, whereas tCys was lowest in the TT group (Table 1). Plasma cystathionine (but not tCys) was higher in men than in women [t (5th, 95th percentiles) concentrations: 0.257 (0.099–0.580) and 0.218 (0.086–0.471) μmol/L, respectively] (P < 0.001). Cystathionine was inversely related to folate, but not to cobalamin or riboflavin, and was positively related to creatinine and methionine (Table 2).

We investigated the relations of cystathionine to the vitamin B-6 vitamers by using a multiple regression model similar to that described for tHcy but with additional adjustment for methionine. Plasma cystathionine increased (P for trend ≤ 0.007) with

### Table 2
Partial Spearman correlation coefficients

<table>
<thead>
<tr>
<th></th>
<th>tHcy</th>
<th>Cystathionine</th>
<th>tCys</th>
<th>PLP</th>
<th>PL</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystathionine</td>
<td>0.15</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>tCys</td>
<td>0.37</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PLP</td>
<td>−0.23</td>
<td>−0.11</td>
<td>0.07</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PL</td>
<td>−0.20</td>
<td>−0.16</td>
<td>0.10</td>
<td>0.80</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PA</td>
<td>−0.21</td>
<td>−0.10</td>
<td>0.10</td>
<td>0.67</td>
<td>0.79</td>
<td>—</td>
</tr>
<tr>
<td>Folate</td>
<td>−0.44</td>
<td>−0.20</td>
<td>0.13</td>
<td>0.39</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Cobalamin</td>
<td>−0.24</td>
<td>−0.02</td>
<td>0.08</td>
<td>0.18</td>
<td>0.14</td>
<td>0.18</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>−0.18</td>
<td>0.003</td>
<td>0.09</td>
<td>0.35</td>
<td>0.39</td>
<td>0.45</td>
</tr>
<tr>
<td>Methionine</td>
<td>−0.07</td>
<td>0.33</td>
<td>−0.02</td>
<td>0.16</td>
<td>0.11</td>
<td>0.07</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.20</td>
<td>0.19</td>
<td>0.16</td>
<td>0.07</td>
<td>0.08</td>
<td>0.19</td>
</tr>
</tbody>
</table>

1 Adjusted for age, sex, and study center, n = 10 601. tHcy, total homocysteine; tCys, total cysteine; PLP, pyridoxal 5’-phosphate; PL, pyridoxal; PA, pyridoxic acid. All correlations were significant if not indicated otherwise, P < 0.001.

2 P = 0.09.

3 P = 0.7.

4 P = 0.016.

### Table 3
Difference in plasma total homocysteine across quartiles (Q) of vitamin B-6 vitamers and MTHFR 677C→T genotypes

<table>
<thead>
<tr>
<th>Determinant</th>
<th>Upper cutoff</th>
<th>All genotypes</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 10 576)</td>
<td>(n = 5 542)</td>
<td>(n = 4 299)</td>
<td>(n = 8 50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q1</td>
<td>32.6</td>
<td>0.73 (0.52, 0.95)</td>
<td>0.57 (0.35, 0.79)</td>
<td>0.54 (0.21, 0.87)</td>
<td>2.18 (0.64, 3.72)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Q2</td>
<td>48.0</td>
<td>0.18 (−0.02, 0.38)</td>
<td>0.25 (0.05, 0.46)</td>
<td>0.10 (−0.20, 0.40)</td>
<td>−0.39 (−1.79, 1.01)</td>
<td></td>
</tr>
<tr>
<td>Q3</td>
<td>73.1</td>
<td>0.09 (−0.10, 0.28)</td>
<td>0.17 (−0.03, 0.36)</td>
<td>0.09 (−0.19, 0.37)</td>
<td>−0.46 (−1.81, 0.89)</td>
<td></td>
</tr>
<tr>
<td>P&lt;0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.003</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q1</td>
<td>7.5</td>
<td>0.22 (0.01, 0.43)</td>
<td>0.06 (−0.16, 0.28)</td>
<td>0.01 (−0.32, 0.33)</td>
<td>2.03 (0.60, 3.46)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Q2</td>
<td>10.0</td>
<td>−0.14 (−0.34, 0.06)</td>
<td>−0.09 (−0.30, 0.12)</td>
<td>−0.10 (−0.41, 0.20)</td>
<td>−0.46 (−1.85, 0.93)</td>
<td></td>
</tr>
<tr>
<td>Q3</td>
<td>14.7</td>
<td>0.00 (−0.19, 0.20)</td>
<td>0.09 (−0.12, 0.29)</td>
<td>0.00 (−0.29, 0.29)</td>
<td>0.10 (−1.24, 1.44)</td>
<td></td>
</tr>
<tr>
<td>P&lt;0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.89</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q1</td>
<td>15.2</td>
<td>0.34 (0.13, 0.56)</td>
<td>0.29 (0.07, 0.50)</td>
<td>0.34 (0.014, 0.66)</td>
<td>1.41 (−0.07, 2.89)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Q2</td>
<td>20.4</td>
<td>0.03 (−0.18, 0.23)</td>
<td>0.11 (−0.10, 0.32)</td>
<td>−0.01 (−0.32, 0.30)</td>
<td>0.03 (−1.41, 1.47)</td>
<td></td>
</tr>
<tr>
<td>Q3</td>
<td>51.7</td>
<td>0.04 (−0.16, 0.23)</td>
<td>0.05 (−0.16, 0.25)</td>
<td>0.01 (−0.29, 0.31)</td>
<td>0.23 (−1.10, 1.55)</td>
<td></td>
</tr>
<tr>
<td>P&lt;0.001</td>
<td>&lt; 0.001</td>
<td>0.002</td>
<td>0.007</td>
<td>0.05</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

1 Comparison of mean values (and 95% CIs) between the highest (referent) quartile (Q4) and each of the other quartiles. MTHFR, methylenetetrahydrofolate reductase; PLP, pyridoxal 5’-phosphate; PL, pyridoxal; PA, pyridoxic acid. Data were obtained by multiple regression with total homocysteine as the dependent variable. The models were adjusted for age, sex, study center, and concentrations of folate, cobalamin, riboflavin, and creatinine.

2 P for interaction between MTHFR 677C→T genotype and vitamin B-6 vitamer.

3 P for trend across quartiles of vitamin B-6 vitamers.
decreasing concentration of PLP, pyridoxal, or PA when investigated in the entire study population (Table 4). There was no significant vitamin B-6 vitamer × genotype interaction (P for interaction > 0.24). Notably, the dose-response relation was different from that observed with tHcy, in that cystathionine concentration decreased throughout the concentration range of PLP, pyridoxal, and PA.

We also investigated the possible relations of tCys to PLP by using multiple regression after adjustment for other B vitamins, creatinine, study center, age, and sex. No such relation was observed (data not shown; P for trend = 0.59).

DISCUSSION

We measured the concentrations of various vitamin B-6 species in human plasma and assessed their relation to the metabolites involved in transsulfuration—homocysteine, cystathionine, and cysteine—in a large population of healthy adults. We found strong correlations between the 3 major vitamin B-6 vitamers—PLP, pyridoxal, and PA—all of which showed a relation to other B vitamins, in particular folate and riboflavin. Vitamin B-6 vitamers, especially PLP, were inversely related to tHcy and cystathionine but not to tCys.

Vitamin B-6

We detected PLP, pyridoxal, and PA in all of the plasma samples, and these vitamer concentrations were strongly correlated. Pyridoxine and pyridoxamine were found in 1.9% and 0.9% of the samples, respectively. The very high PLP (10, 11), pyridoxal (10, 11, 58), and PA (10, 11, 58) concentrations observed in some samples are most likely caused by the recent intake of high doses of vitamin B-6, although we do not have vitamin supplementation data to verify that possibility. Nonfasting populations are expected to show a greater variation in vitamin B-6 concentrations than are fasting populations, because higher vitamin B-6 concentrations may be attained after a recent meal containing vitamin B-6 and also after the ingestion of a vitamin supplement, which sometimes accompanies a meal. The large variation in PLP, pyridoxal, PA, and pyridoxine at high total vitamin B-6 concentrations could be explained by variable vitamin B-6 intakes and the incomplete conversion of pyridoxine to other forms after recent supplementation because the conversion of pyridoxine to other vitamin B-6 forms takes a few hours (5, 6, 58). The presence of pyridoxamine in some samples was always accompanied by very high PLP, pyridoxal, and PA concentrations and sometimes also by high pyridoxine concentrations, which suggests that it is related to a recent intake of a supplement containing pyridoxine. The faster and stronger increases in plasma concentrations of pyridoxal and PA than in those of PLP that are induced by recent vitamin B-6 supplementation (2, 5, 7) may explain the increased strength of PLP-pyridoxal and PLP-PA to relations with increasing PLP concentrations (Figure 1).

The 3 main vitamin B-6 species showed a moderate correlation with the concentrations of other B vitamins, in particular folate and riboflavin. This correlation is probably due to overlapping dietary sources of these 3 B vitamins, including fruit and vegetables (59). The weak association with cobalamin is probably explained by the fact that cobalamin is mainly derived from food items other than fruit and vegetables—primarily, animal products (59).

Of the vitamin B-6 vitamers, PA showed the strongest relation to other B vitamins, in particular folate, cobalamin, riboflavin, creatinine and methionine. Notably, the dose-response relation was different from that observed with tHcy, in that cystathionine but not to tCys.

The 3 main vitamin B-6 species showed a moderate correlation with the concentrations of other B vitamins, in particular folate and riboflavin. This correlation is probably due to overlapping dietary sources of these 3 B vitamins, including fruit and vegetables (59). The weak association with cobalamin is probably explained by the fact that cobalamin is mainly derived from food items other than fruit and vegetables—primarily, animal products (59).

Of the vitamin B-6 vitamers, PA showed the strongest relation to creatinine. This finding is in agreement with published data showing that PA is sensitive to renal function (23) and that it accumulates during renal failure (60).
The associations of vitamin B-6 vitamers with other B vitamins and renal function indicate that these factors are potential confounders in investigations of the relation of vitamin B-6 status and clinical outcomes or metabolite concentrations. It has been suggested that the ratio of PA to pyridoxal can distinguish between increases in PA concentrations that are due to increased dietary intake and those that are due to renal impairment (60).

**Homocysteine**

The influence of vitamin B-6 on tHcy is moderate in this study and is present only at vitamer concentrations in the lowest quartile, which agrees with findings of a previous study (25). We also found that this relation was strongest for PLP and pyridoxal in the TT group. The genotype effects may explain why most authors report no PLP-tHcy relation (27–35). This also agrees with the fact that most studies report no effect of vitamin B-6 supplementation on fasting plasma tHcy concentrations (29, 33, 38–45).

PLP serves as the cofactor of cystathionine β-synthase (24), which could partly explain the inverse relation between vitamin B-6 and plasma tHcy. Vitamin B-6 nutrition may also affect homocysteine status by influencing the folate-metabolizing enzyme serine hydroxymethyltransferase (61).

**Cystathionine and cysteine**

All 3 major vitamin B-6 forms—in particular, PLP and pyridoxal—were inversely related to cystathionine concentrations. This suggests that cystathionine degradation catalyzed by cystathionine γ-lyase is the rate-limiting step in transsulfuration. Cystathionine was found to be inversely related to the concentration of the major B-6 vitamer forms throughout their concentration ranges, which is consistent with the linear relation of PLP concentrations to cystathionine γ-lyase activity in the liver of rats (62). Thus, the dose response differed from that observed for tHcy, which increased only at low vitamin B-6 vitamer concentrations. Such differences between the relations of vitamin B-6 status to homocysteine and to cystathionine are in accordance with the greater sensitivity of cystathionine γ-lyase enzyme than of cystathionine β-synthase to vitamin B-6 status (35, 62–64).

Plasma concentrations of cystathionine increase (65) and those of PLP decrease (66, 67) in the hours after the consumption of proteins, and recent protein intake may therefore enhance the inverse relation of vitamin B-6 to cystathionine. Conversely, vitamin B-6 intake, either from food or vitamin supplement, may counteract this short-term effect.

In accordance with published reports, we observed no relation between PLP and the concentration of tCys (23, 35, 47). However, this observation allows no inference about the role of transsulfuration in cysteine homeostasis, partly because tCys is mainly protein-bound in plasma and undergoes complex displacement and disulfide exchange reactions with homocysteine (68). Furthermore, cysteine is a component of dietary protein and is obtained from food.

**MTHFR 677C→T polymorphism**

We observed that plasma tHcy increased and folate decreased according to the number of MTHFR 677T alleles. A folate × MTHFR 677C→T genotype interaction as a determinant of plasma tHcy has been shown in numerous studies (48, 69). The association of PLP (70), pyridoxal, and PA with tHcy is modified by the MTHFR 677C→T genotype. Thus, vitamin B-6 shares this effect modification with other nonfolate B vitamins involved in homocysteine metabolism, including riboflavin (69) and cobalamin (71). A likely explanation is that impaired 5-methyltetrahydrofolate formation and homocysteine remethylation in the TT genotype direct homocysteine to the transsulfuration pathway.

Of the vitamin B-6 vitamers, only PLP had its lowest concentrations in subjects with the TT genotype, and this difference between genotypes was modest compared with that found for folate in these subjects. One may speculate whether a lower PLP concentration reflects a greater flux through the transsulfuration pathway in subjects with the TT genotype. Likewise, it has been speculated that greater metabolic activity decreases the concentrations of cofactors involved, including vitamin B-6 (72).

Neither the cystathionine concentration nor the relation of vitamin B-6 to cystathionine was modified by the MTHFR 677C→T genotype. This finding agrees with the fact that MTHFR and related folate species are not involved in cystathionine metabolism (24).

**Conclusions**

In this study, we showed that plasma concentrations of the main vitamin B-6 vitamers were strongly correlated but also had a moderate association with other B vitamins, in particular folate and riboflavin, that was due to overlapping dietary sources of these vitamins. The population size was large enough to provide precise estimates of the metabolic effects of differences in vitamin B-6 status on the plasma concentrations of tHcy and cystathionine. These associations were in accordance with experimental data on the role of PLP as a cofactor for cystathionine β-synthase and cystathionine γ-lyase. PLP and pyridoxal had the strongest association with these transsulfuration metabolites, which may reflect the role of PLP as cofactor and the ability of pyridoxal to cross cell membranes (3, 14, 15). The inverse relation between PLP and tHcy was strongest and the PLP concentration was lowest in subjects with the MTHFR 677TT genotype, possibly because of impaired homocysteine remethylation and increased flux through the transsulfuration pathway. Thus, the present study shows that the transsulfuration metabolites in humans reflect the role of vitamin B-6 as a cofactor for cystathionine β-synthase and cystathionine γ-lyase.

We thank Geir Hoff and Trygve Grotmol at The Cancer Registry of Norway for organizing the study and the technical staff at the Section for Pharmacology for analyzing the plasma samples.

The authors’ responsibilities were as follows—JS and PMU: study design; ØM, SH, SEV, and PMU: data management, statistical analysis, and interpretation of the data; ØM, PMU, and SH: writing of the manuscript; and SEV and JS: review of the manuscript. PMU has received consulting fees from Nycomed and is a member of the steering board of the nonprofit Foundation to Promote Research into Functional Vitamin-B12 Deficiency. None of the other authors had a personal or financial conflict of interest.

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Folate, but not vitamin B-12 status, predicts respiratory morbidity in north Indian children

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ABSTRACT
Background: Vitamin deficiencies are often part of malnutrition, which predisposes to acute lower respiratory tract infections.
Objective: The objective was to measure the association between cobalamin and folate status and subsequent respiratory morbidity.
Design: A prospective cohort study was conducted in 2482 children aged 6–30 mo nested in a zinc supplementation trial. We measured plasma concentrations of folate, cobalamin, methylmalonic acid, and total homocysteine (tHcy) and followed the children for 4 mo.
Results: We observed 1176 episodes of acute lower respiratory tract infections. Children with folate concentrations in the lowest quartile (interquartile range: 6.4–20.0 nmol/L) had a 44% higher incidence [adjusted incidence rate ratio (IRR): 1.44; 95% CI: 1.23, 1.70] of acute lower respiratory tract infections than did children in the other 3 quartiles. For tHcy, the IRR was 1.24 (1.07, 1.40) in a comparison of those in the highest quartile with those in the other quartiles. Breastfeeding was associated with high folate concentrations and protection against subsequent respiratory tract infections. This protection was significantly and substantially reduced after adjustment for plasma folate concentrations at baseline. Compared with the children in the other 3 quartiles, the IRR for being in the lowest quartile of cobalamin was 1.13 (0.76, 1.03) and for being in the highest quartile of methylmalonic acid was 1.12 (0.96, 1.31).
Conclusions: Poor folate status appears to be an independent risk factor for lower respiratory tract infections in young children. This study also suggests that the protective effect of breastfeeding is partly mediated by folate provided through breast milk. Am J Clin Nutr 2007;86:139–44.

KEY WORDS Children, pneumonia, folate, cobalamin, homocysteine, methylmalonic acid, malnutrition, cohort study, India

INTRODUCTION

In children in developing countries, acute lower respiratory tract infections are among the most common causes of death, claiming ∼2 million lives every year (1). Known risk factors are young age, low birth weight, pollutants, poverty, malnutrition, zinc deficiency, and lack of breastfeeding (2). Therapeutic or prophylactic administration of zinc to young children reduces the risk of acute lower respiratory tract infections and the episode duration (3–6). Whether deficiencies of other nutrients, such as folate or vitamin B-12 (cobalamin), are independent risk factors for lower respiratory tract infections, is not known. The main sources of cobalamin are animal products, of which poor children have a low intake (7). It is therefore plausible that many children of developing countries are cobalamin deficient. Folate deficiency, however, is presumably less prevalent because of the abundance of folate in breast milk and because of the predominantly vegetarian diet in many low-income countries, such as in South Asia (8).

We undertook a prospective cohort study to assess whether poor folate and poor cobalamin status were risk factors for acute lower respiratory tract infections in young children.

SUBJECTS AND METHODS

Study population

This study was nested within a zinc supplementation trial in 2482 children aged 6–30 mo. Assessment of the association between markers of cobalamin and folate status and respiratory illness were predefined secondary objectives in this project. The inclusion and exclusion criteria and the effects of zinc administration are described elsewhere (3). The ethics committee of the All India Institute of Medical Sciences in New Delhi approved the study. Details of the study were given in writing and were also

1 From the Centre for International Health (TAS, NB, and HS) and the Section for Pharmacology, Institute of Medicine (PMU and JS), University of Bergen, Bergen, Norway; the All India Institute of Medical Sciences, New Delhi, India (ST and MKB); the Society for Applied Studies, Kolkata, India (ST and MB); the Institute of Basic Medical Sciences, Department of Nutrition, University of Oslo, Oslo, Norway (HR); the Oxford Centre for Gene Function, Department of Physiology, Anatomy & Genetics, Oxford University, Oxford, United Kingdom (HR); the Norwegian Institute of Public Health, Oslo, Norway (HKG); the World Health Organization, Geneva, Switzerland (RB); the Department of Medical Biosciences, Clinical Chemistry, University of Umeå, Umeå, Sweden (JS); and the Department of Biotechnology, New Delhi, India (MBK).

2 The sponsors of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all of the data in the study and had final responsibility for the decision to submit for publication.

3 Supported by grants from the European Commission (EU- INCO-DC contract number IC18-CT96-0045 and INCO-FP6-003740), the Norwegian Research Council, the Norwegian Advanced Research Programme (NRC project no 164301/V40), and the Norwegian Council of Universities’ Committee for Development Research and Education (NUFU project number PRO 52-53/96 and 36/2002).

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read to the parents in the presence of a witness. Signatures or thumb impressions were obtained on a consent form.

Data collection

A study physician interviewed the caretaker, examined the child, and collected a venous blood specimen from the child on enrollment. Fieldworkers visited each child every seventh day for 4 mo. At each visit, the caretakers were asked about fever, cough, and other symptoms of disease and whether they had sought treatment for the child in the previous 7 d. Respiratory rates were counted twice for 1 min, and the temperature was recorded. The caretakers were encouraged to bring their children to the study clinic whenever they were ill. In the field clinic, 2 physicians assessed the child.

Acute lower respiratory tract infection was defined by cough and fast breathing or lower chest indrawing. Fast breathing was defined as 2 counts of ≥50 breaths/min for infants (<12 mo of age) and ≥40 breaths/min for older children. We defined 2 episodes to be distinct from each other when they were separated by >14 continuous days without acute lower respiratory tract infection.

Treatment

Acute illness was treated according to the guidelines of the World Health Organization. Children with acute lower respiratory tract infections received co-trimoxazole, which was substituted with amoxicillin if the child did not respond within 3 d. Acute lower respiratory tract infections in 2482 Indian children aged 6–30 mo was summarized for each child; these summary measures were used as outcome variables. We used negative binomial regression to estimate the incidence rate ratio (IRR) for acute lower respiratory tract infections between categories or units of the exposure variables. The negative binomial distribution was used instead of the Poisson distribution because there was overdispersion in the data. Exposure variables were plasma concentrations of folate, cobalamin, tHcy, and methylmalonic acid at the day of inclusion. These variables were included as dichotomous or continuous variables in generalized linear models and continuous variables in generalized additive regression models with the negative binomial distribution family and a logarithmic link function. We used log (base 2)-transformed values of the exposure variables when they were right-skewed and when entered as continuous variables in the regression models. The exposure variables were also dichotomized into categories above or below the 25th percentile for folate and cobalamin and into categories above or below the 75th percentile for tHcy and methylmalonic acid. We identified predictors for acute lower respiratory tract infections in a stepwise process. We assessed whether these and several other variables confounded the association between folate, tHcy, vitamin B-12, or methylmalonic acid concentrations and respiratory tract infections by adding them to the multiple model one at a time. These variables were height-for-age, weight-for-age, and weight-for-length z scores, age, breastfeeding status, sex, season, zinc supplementation status, family type [nuclear or joint (multigenerational)], family size, income, and years of schooling of the mothers and fathers (Table 1).

Blood collection and biochemical analyses

We collected samples of nonfasting venous blood (~5 mL) in heparinized polypropylene tubes (Sarstedt, Nümbrecht, Germany) between 0900 and 1200. The samples were centrifuged (447 × g, 10 min, room temperature), and the plasma was divided and stored into polypropylene vials (Eppendorf, Hinz, Germany) at −20 °C until analyzed. All samples were analyzed at the University of Bergen, Bergen, Norway (Section for Pharmacology, Institute of Medicine). Plasma cobalamin and plasma folate concentrations were estimated by microbiological assays with the use of a chloramphenicol-resistant strain of Lactobacillus casei and a colistin sulfate-resistant strain of Lactobacillus leichmannii, respectively (9, 10). Both assays were adapted to a microtiter plate format and carried out by a robotic workstation (11). Plasma methylmalonic acid and total homocysteine (tHcy) were analyzed with a modified gas chromatography–mass spectrometry method based on ethylchloroformate derivatizations (12).

Data management and statistical analyses

The data entry forms were designed with FOXPRO for WINDOWS (Microsoft Corporation, Redmond, WA), with range and consistency checks incorporated. Double data entry by 2 data encoders followed by validation was completed within 48 h after the forms were completed in the field. Growth was assessed by calculating height-for-age and weight-for-age, and weight-for-height z scores based on the 1978 references with the use of EPIINFO 6 (13, 14). Summary measures for continuous variables are reported as means or medians as appropriate, and categorical variables are reported as proportions. We used the Mann–Whitney U nonparametric test to compare the plasma concentration of folate, tHcy, cobalamin, and methylmalonic acid between age and breastfeeding categories. The number of episodes of acute lower respiratory tract infections was summarized for each child; these summary measures were used as outcome variables. We used negative binomial regression to estimate the incidence rate ratio (IRR) for acute lower respiratory tract infections between categories or units of the exposure variables. The negative binomial distribution was used instead of the Poisson distribution because there was overdispersion in the data. Exposure variables were plasma concentrations of folate, cobalamin, tHcy, and methylmalonic acid at the day of inclusion. These variables were included as dichotomous or continuous variables in generalized linear models and continuous variables in generalized additive regression models with the negative binomial distribution family and a logarithmic link function. We used log (base 2)-transformed values of the exposure variables when they were right-skewed and when entered as continuous variables in the regression models. The exposure variables were also dichotomized into categories above or below the 25th percentile for folate and cobalamin and into categories above or below the 75th percentile for tHcy and methylmalonic acid. We identified predictors for acute lower respiratory tract infections in a stepwise process. We assessed whether these and several other variables confounded the association between folate, tHcy, vitamin B-12, or methylmalonic acid concentrations and respiratory tract infections by adding them to the multiple model one at a time. These variables were height-for-age, weight-for-age, and weight-for-length z scores, age, breastfeeding status, sex, season, zinc supplementation status, family type [nuclear or joint (multigenerational)], family size, income, and years of schooling of the mothers and fathers (Table 1). Statistical analyses were done with STATA 9.0 sta-

| Variables assessed in the multivariable regression models that measured the association between plasma folate, cobalamin, homocysteine, or methylmalonic acid and the subsequent incidence of acute respiratory tract infections in 2482 Indian children aged 6–30 mo |
|---------------------------------|-------------------|-------------------|
| **Continuous**                  | **Categorical**   |
| Breastfeeding                   | —                 | Yes or no         |
| Age                             | Months            | Infants: yes or no|
| Sex                             | —                 | Male or female    |
| Living in multigenerational     | —                 | Yes or no         |
| families                        |                   |                   |
| Members in the household        | Number            |                   |
| Years of schooling, mother      | Years             | School: yes or no |
| Years of schooling, father      | Years             | School: yes or no |
| Weight-for-age z score          | z scores          | ≤−2 WAZ: yes or no|
| Weight-for-length z score       | z scores          | ≤−2 WHZ: yes or no|
| Height-for-age z score          | z scores          | ≤−2 HAZ: yes or no|
| Maternal age                    | Years             |                   |
| Paternal age                    | Years             |                   |
| Income                          | 1000 Indian rupees|                   |
| Time since blood sampling       | Days              |                   |
| Season                          | —                 | 3 Categories      |
| Zinc supplementation status     | —                 | Given: yes or no  |

1 Interactions assessed: of age with breastfeeding, of age with sex, and of folate, homocysteine, cobalamin, and methylmalonic acid with breastfeeding status and age categories.
FOLATE AND CHILDHOOD RESPIRATORY INFECTIONS

TABLE 2
Baseline characteristics of the children in the cohort by breastfeeding status and age*

<table>
<thead>
<tr>
<th>Variable</th>
<th>6–11 mo</th>
<th>12–30 mo</th>
<th>6–11 mo</th>
<th>12–30 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects [n (%)]</td>
<td>854 (34.4)</td>
<td>869 (35.0)</td>
<td>152 (6.1)</td>
<td>607 (24.5)</td>
</tr>
<tr>
<td>Boys [n (%)]</td>
<td>461 (53.9)</td>
<td>465 (53.5)</td>
<td>79 (52.0)</td>
<td>295 (48.6)</td>
</tr>
<tr>
<td>Weight-for-length z score</td>
<td>$-0.65 \pm 0.9^2$</td>
<td>$-1.52 \pm 0.8$</td>
<td>$-1.06 \pm 0.9$</td>
<td>$-1.35 \pm 0.8$</td>
</tr>
<tr>
<td>Weight-for-length z score &lt; −2 [n (%)]</td>
<td>261 (30.6)</td>
<td>559 (64.3)</td>
<td>76 (50.0)</td>
<td>349 (57.5)</td>
</tr>
<tr>
<td>Length-for-age z score</td>
<td>$-1.27 \pm 0.9$</td>
<td>$-1.94 \pm 1.1$</td>
<td>$-1.64 \pm 1.1$</td>
<td>$-1.83 \pm 1.2$</td>
</tr>
<tr>
<td>Living-for-age z score &lt; −2 [n (%)]</td>
<td>177 (20.7)</td>
<td>392 (45.1)</td>
<td>54 (35.5)</td>
<td>251 (41.4)</td>
</tr>
<tr>
<td>Income (in 1000 rupees)</td>
<td>47.2 ± 42.0</td>
<td>44.7 ± 46.8</td>
<td>45.1 ± 32.1</td>
<td>45.2 ± 37.2</td>
</tr>
<tr>
<td>Duration of schooling, mother (y)</td>
<td>5.2 ± 4.5</td>
<td>5.0 ± 4.5</td>
<td>5.4 ± 4.4</td>
<td>5.4 ± 4.7</td>
</tr>
<tr>
<td>Duration of schooling, father (y)</td>
<td>8.7 ± 4.0</td>
<td>8.2 ± 4.4</td>
<td>8.5 ± 3.7</td>
<td>8.5 ± 4.1</td>
</tr>
<tr>
<td>No. of individuals in household (n)</td>
<td>5.9 ± 2.3</td>
<td>5.8 ± 2.4</td>
<td>6.1 ± 2.7</td>
<td>5.9 ± 2.1</td>
</tr>
<tr>
<td>Living in multigenerational families [n (%)]</td>
<td>449 (52.6)</td>
<td>383 (44.0)</td>
<td>89 (58.6)</td>
<td>310 (51.1)</td>
</tr>
<tr>
<td>Cobalamin (pmol/L)</td>
<td>184 (120–263)$^3$</td>
<td>172 (124–253)</td>
<td>334 (235–463)</td>
<td>261 (194–348)</td>
</tr>
<tr>
<td>Folate (nmol/L)$^5$</td>
<td>20.2 (11.7–34.4)</td>
<td>11.3 (7.4–17.6)</td>
<td>5.3 (3.4–7.7)</td>
<td>6.5 (4.7–9.2)</td>
</tr>
<tr>
<td>tHcy (μmol/L)$^6$</td>
<td>12.6 (9.2–18.1)</td>
<td>11.3 (8.7–15.2)</td>
<td>10.7 (8.2–13.9)</td>
<td>9.1 (7.4–11.2)</td>
</tr>
<tr>
<td>MMA (μmol/L)$^7$</td>
<td>1.03 (0.54–2.08)</td>
<td>0.74 (0.42–1.36)</td>
<td>0.45 (0.31–0.71)</td>
<td>0.38 (0.26–0.59)</td>
</tr>
</tbody>
</table>

* tHcy, total homocysteine; MMA, methylmalonic acid.

1 tHcy, total homocysteine; MMA, methylmalonic acid.
2 $\bar{x} \pm$ SD (all such values).
3 $P$ values were calculated by using the Mann-Whitney $U$ nonparametric test. $P$ values were < 0.001 for the differences in cobalamin, folate, tHcy, and MMA concentrations between breastfeeding categories in infants and toddlers (children aged ≥12 mo) and between infants and toddlers who were breastfed and those who were not breastfed.
4 $P = 0.36$ for the comparison of cobalamin concentrations between infants and toddlers who were breastfed.
5 Median; interquartile range in parentheses (all such values).
6 $P = 0.002$ for the comparison of concentrations of MMA between infants and toddlers who were not breastfed.

RESULTS
Demographics and blood indexes
Selected baseline characteristics of the study subjects according to breastfeeding status and age categories are shown in Table 2. The mean (±SD) age of the subjects was 15.3 ± 7.5 mo. Fifty-two percent of the subjects were boys, and 69% were breastfed. The median plasma concentrations of cobalamin, folate, tHcy, and methylmalonic acid were 205 (IQR: 141–299) pmol/L, 10.7 (6.4–20.0) nmol/L, 10.9 (8.3–14.9) μmol/L, and 0.65 (0.37–1.30) μmol/L, respectively. The folate concentration was substantially and significantly lower in children that were not breastfed than in those who were breastfed (Table 2).

Risk factors for acute lower respiratory tract infections
During a total of 266 537 d of follow-up, there were 848 children who had 1176 episodes of acute lower respiratory tract infections: 69% of these children had only one episode, 25% had 2 episodes, and 6% had >2 episodes. Not being breastfed, young age, being enrolled in the wet or cool months (June to January) compared with the months of February to May, and a low length-for-age z score were all independent risk factors (Figure 1, Table 3). Socioeconomic factors, however, were not associated with acute lower respiratory tract infections.

Folate and cobalamin status and acute lower respiratory tract infections
In the crude analyses, low folate and high tHcy concentrations were predictors for acute lower respiratory tract infections, whereas low cobalamin and high methylmalonic acid concentrations were not (Table 4). After adjustment for potential confounders (listed in Table 1), the associations of folate and tHcy with respiratory tract infections were maintained, whereas plasma methylmalonic acid and cobalamin concentrations remained unassociated with respiratory morbidity (Table 4). Furthermore, methylmalonic acid or cobalamin and acute lower respiratory tract infections were still not associated after inclusion of folate or tHcy concentration in the statistical models.

To avoid overlooking nonlinear associations, we also undertook multivariable analyses with these markers as smooth terms in generalized additive models. In these models, with adjustment for the same variables, both plasma folate (Figure 2) and tHcy were predictors of acute lower respiratory tract infections. The inverse association between log IRR and folate concentration was linear at a folate concentration < 20 nmol/L (the lowest 3
Children aged 6–30 mo who were followed for 4 mo

Predictors of acute lower respiratory tract infection in 2482 Indian nonbreasted and breastfed infants and children aged 12–30 mo with and without adjustment for plasma folate concentration at enrollment. To estimate the extent to which the effect of breastfeeding was altered after adjustment for plasma folate concentration, we performed a nonparametric bootstrap analysis (16). The estimate for breastfeeding was attenuated 1.34-fold (95% CI: 1.04, 1.76; P = 0.011) and 1.17-fold (95% CI: 1.03, 1.32; P = 0.008) in infants and older children, respectively.

In contrast, there was a linear and positive association between the incidence of respiratory tract infections and tHcy concentrations throughout the whole range of tHcy concentrations. The results from the multivariable generalized additive models confirmed that there were no associations between baseline methylmalonic acid or cobalamin and subsequent acute lower respiratory tract infections.

Children with a folate concentration in the lowest quartile had a 44% (95% CI: 23%, 70%) higher incidence of respiratory tract infections than did those with a folate concentration in the highest 3 quartiles (Table 4). The IRR of acute lower respiratory tract infections was 0.90 (95% CI: 0.84, 0.96) for each doubling [one-unit increase in the log transformed (base 2) value] of folate concentration. Thus, the higher the folate concentration, the lower the incidence.

Those with a tHcy concentration in the highest quartile had a 24% (95% CI: 7%, 40%) (Table 4) higher incidence than did those with lower concentrations of tHcy. Furthermore, for each doubling in tHcy concentration, the incidence increased by 22% (95% CI: 10%, 36%). Thus, the higher the tHcy concentration, the higher the incidence of acute lower respiratory tract infections.

Inclusion of both folate and tHcy in the models did not alter the estimate of the other compared with when either folate or tHcy was included in the multivariable negative binomial regression model. Furthermore, in the multivariable regression models and in stratified analyses, the associations of acute lower respiratory tract infections with plasma folate, cobalamin, tHcy, or methylmalonic acid were not modified by zinc administration or breastfeeding status. The protective effect of breastfeeding on respiratory tract infections was substantially and significantly reduced when the folate concentration was added to the regression models, particularly in infants (Figure 1).

The effect of poor cobalamin status on plasma folate

In this predominant vegetarian population, the plasma folate concentration may have been high because of cobalamin deficiency, which led to the folate trap phenomenon (17, 18). This may obscure the assessment of folate status in cobalamin-deficient subjects. We therefore depicted the association between plasma cobalamin and plasma folate from continuous generalized additive models. This analysis showed that the plasma folate concentration started to increase when the cobalamin concentration was <250 pmol/L. We therefore investigated the relation between acute lower respiratory tract infections and folate in a subgroup with presumably adequate cobalamin status, ie, plasma cobalamin >250 pmol/L. For these children, the adjusted IRR was 1.53 (95% CI: 1.22, 1.93) in a comparison of the lower (lower 25%) and upper (upper 75%) 3 quartiles of plasma folate. Thus, the association between folate and respiratory tract infections was not significantly different between the subgroups consisting of children with a cobalamin concentration ≤250 pmol/L and those with higher cobalamin concentrations (P for interaction = 0.2)

**DISCUSSION**

Our analyses showed a strong and independent association between low plasma folate concentrations and the risk of acute lower respiratory tract infections. Children in the lowest quartile of plasma folate had an almost 50% greater risk than did the other children. The association between acute lower respiratory tract infections and folate was not altered by adjustment for other risk factors for acute lower respiratory tract infections or other potential confounders that we measured.

Children who were not breastfed had a significantly lower plasma folate concentration than did breastfed children of the same age. This difference was larger in the lowest age categories. In fact, breastfed infants had a median folate concentration that was almost 4 times that of infants who were not breastfed. Our analyses also showed that children who were breastfed had a lower incidence of acute lower respiratory tract infections than did children who were not breastfed. The effect of breastfeeding on respiratory illness was significantly and substantially reduced when the folate concentration was included in the regression.

**FIGURE 1.** Adjusted odds ratios for having ≥1 episode of an acute respiratory tract infection between 2482 Indian nonbreasted and breastfed infants and children aged 12–30 mo with and without adjustment for plasma folate concentration at enrollment. To estimate the extent to which the effect of breastfeeding was altered after adjustment for plasma folate concentration, we performed a nonparametric bootstrap analysis (16). The estimate for breastfeeding was attenuated 1.34-fold (95% CI: 1.04, 1.76; P = 0.011) and 1.17-fold (95% CI: 1.03, 1.32; P = 0.008) in infants and older children, respectively.

**TABLE 3**

Predictors of acute lower respiratory tract infection in 2482 Indian children aged 6–30 mo who were followed for 4 mo

<table>
<thead>
<tr>
<th>Variable</th>
<th>IRR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per month in breastfed, children</td>
<td>0.96 (0.95, 0.98)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Per month in non-breastfed children</td>
<td>0.99 (0.97, 1.00)</td>
<td>0.015</td>
</tr>
<tr>
<td>Interaction of breastfeeding status with age</td>
<td>0.98 (0.96, 0.99)</td>
<td>0.01</td>
</tr>
<tr>
<td>Season*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>February through May (reference)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>June through September</td>
<td>1.59 (1.36, 1.87)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>October through January</td>
<td>1.45 (1.22, 1.72)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Height-for-age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per z score</td>
<td>0.88 (0.83, 0.93)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

*IRR, incidence rate ratio (calculated by negative binomial regression and adjusted for time of blood sampling, sex, zinc supplementation, and socioeconomic status). The effect of breastfeeding is depicted in Figure 1.

*P for trend across seasons < 0.001.
TABLE 4
Associations between concentrations of the plasma markers of folate and cobalamin status and the incidence of acute lower respiratory tract infections in Indian children aged 6–30 mo.

<table>
<thead>
<tr>
<th>Duration of follow-up</th>
<th>Folate</th>
<th>Cobalamin</th>
<th>Homocysteine</th>
<th>Methylmalonic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reference (quartiles 2–4)</td>
<td>Low (quartile 1)</td>
<td>Reference (quartiles 1–3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>187 668</td>
<td>62 761</td>
<td>186 665</td>
</tr>
<tr>
<td></td>
<td></td>
<td>759</td>
<td>359</td>
<td>783</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.48</td>
<td>2.09</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>Unadjusted IRR (95% CI)</td>
<td>1.42 (1.23, 1.63)</td>
<td>1.08 (0.93, 1.25)</td>
<td>1.22 (1.06, 1.42)</td>
</tr>
<tr>
<td></td>
<td>Adjusted IRR (95% CI)</td>
<td>1.44 (1.23, 1.70)</td>
<td>1.13 (0.76, 1.03)</td>
<td>1.24 (1.07, 1.40)</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>&lt; 0.001</td>
<td>0.13</td>
<td>0.005</td>
</tr>
</tbody>
</table>

1 IRR, incidence rate ratio (calculated by negative binomial regression).
2 Adjusted for age, breastfeeding status, interaction of age with breastfeeding, time of blood sampling, sex, zinc supplementation, nutritional status (height-for-age z score), socioeconomic status (income, family, size of education of parents, and multigenerational or nuclear family), and season.

models. This indicates that the beneficial effect of breast milk may be due in part to its folate content.

We found that the incidence of acute lower respiratory tract infections increased throughout the whole range of tHcy concentrations. In adults, the plasma tHcy concentration is a good marker of folate status, and it increases with declining folate status (19). Because a low plasma folate concentration was found to be a strong predictor of respiratory tract infections, we anticipated that the tHcy effect would be attenuated after adjustment for folate concentration and vice versa. This was, however, not the case, which suggests that tHcy and folate are independent predictors of acute lower respiratory tract infections. tHcy is not a specific marker of folate status (20), and the plasma folate concentration explained little of the variability of tHcy in our study (data not shown). Indeed, in newborns and breastfed infants, low plasma cobalamin concentrations are more important determinants of tHcy than are low folate concentrations (21, 22). It is therefore surprising that tHcy was associated with acute lower respiratory tract infections, whereas plasma concentrations of cobalamin and methylmalonic acid were not. Notably, tHcy increases with activation of the immune system, particularly the TH1 response (23, 24). A high burden of infectious diseases may therefore cause immune activation and elevated tHcy, which may explain the association between tHcy at baseline and subsequent respiratory morbidity.

It is an inherent weakness of cohort studies that they identify associations rather than causality and that the observed associations can be due to confounding. We therefore undertook several multivariable regression analyses with outcomes as dichotomous (logistic) and count (negative binomial) variables, adjusting for socioeconomic, anthropometric, and clinical variables. In these models, the association between folate or tHcy and subsequent morbidity were not attenuated. However, we cannot rule out that the observed association is confounded by variables that we did not measure. For example, children with low folate concentrations could come from homes with poorer indoor air quality, which is a well-known risk factor for childhood pneumonia (2). However, plasma folate concentration at recruitment was still associated with the risk of acute lower respiratory tract infection after adjustment for family size, income, maternal and paternal education, and type of housing, which are probably related to indoor air pollution. Moreover, the plasma folate concentration could be associated with other nutrients, such as vitamin A, that might be associated with respiratory tract infections (2). In any case, our findings need to be verified in clinical trials to have clinical or public health implications.

The folate trap phenomenon might cause high plasma folate but low cellular folate concentrations in cobalamin-deficient individuals (17, 18). However, elevated plasma folate concentrations in children with plasma cobalamin <250 pmol/L would
then attenuate the folate-respiratory tract infections relation rather than produce false results. This assumption is in line with the observation that neither the cobalamin nor the methylmalonic acid concentration was associated with acute lower respiratory tract infections. Furthermore, the increased risk of acute lower respiratory tract infections in children with low folate concentrations did not change when we excluded children with poor cobalamin status from the analysis.

The defense against respiratory tract infections relies on the ability of the immune cells to proliferate and differentiate and on the effective renewal of the respiratory epithelial linings. Folates play a crucial role in DNA and protein synthesis, which suggests that processes in which cell proliferation is essential may be impained by poor folate status. Indeed, macroscopic disruption of the epithelial lining occurs with anti-folate treatment (25), and several facets of the immune system are affected by folate deficiency (25, 26). The phagocytic and bactericidal ability of polymorphonuclear leukocytes is poor in individuals with severe folate deficiency and improves with folate replenishment (27). Furthermore, the thymus and cell-mediated immunity, the blastogenic response of T lymphocytes to certain mitogens, and the antibody responses to several antigens is reduced in folate-deficient individuals (25). These changes to the immune system caused by folate deficiency may result in an increased susceptibility to infections.

Some antimarial drugs act on the folate metabolism of the parasite, and folate administration during sulfadoxine-pyrimethamine prophylaxis has been shown to reduce its efficacy (28). This needs to be kept in mind if folate is given to populations in areas where malaria is endemic and sulfadoxine-pyrimethamine is commonly used.

Little attention has been paid to folate deficiency as a public health problem in children of developing countries. We found that poor folate status was an independent risk factor for acute lower respiratory tract infections and that the beneficial effect of breastfeeding may in part be explained by the high folate concentrations in breast milk. Conceivably, folic acid given in adequate amounts may counteract some of the negative consequences faced by children that cannot be breastfed, such as orphans or children of HIV-infected mothers. These children may be at increased risk of infections, because of poverty, poor nutrition, and poor access to health care. Supplementation or fortification with folic acid may reduce their burden of infection. This hypothesis, however, should be explored in clinical trials before any public health measures are taken.

We thank Elfrid Blondal, Beate Olsen, and Ove Netland for help with the analysis of plasma cobalamin, folate, homocysteine, and methyalmalonic acid.

The authors’ responsibilities were as follows—TAS: participated in the protocol design, planning, and analysis and wrote the first draft of the manuscript; ST, NB, MKB, and HS (overall coordinator of the project): participated in the design, field implementation, data management and analysis, and preparation of the manuscript; HR and PMU: participated in the planning, biochemical analyses, statistical analyses, and preparation of the manuscript; HKG: participated in the statistical analyses; RB (staff member of the WHO and is alone responsible for the views expressed herein, which do not necessarily represent the decisions, policy, or views of the WHO): participated in the planning, design, and data management; JS: participated in the planning and biochemical analyses. All authors approved the final version of the manuscript.

REFERENCES


Human adrenal glands secrete vitamin C in response to adrenocorticotrophic hormone

Sebastian J Padayatty, John L Doppman, Richard Chang, Yaohui Wang, John Gill, Dimitris A Papanicolaou, and Mark Levine

ABSTRACT

Background: When vitamin C intake is from foods, fasting plasma concentrations do not exceed 80 μmol/L. We postulated that such tight control permits a paracrine function of vitamin C.

Objective: The purpose of this study was to determine whether paracrine secretion of vitamin C from the adrenal glands occurs.

Design: During diagnostic evaluation of 26 patients with hyperaldosteronism, we administered adrenocorticotrophic hormone intravenously and measured vitamin C and cortisol in adrenal and peripheral veins.

Results: Adrenal vein vitamin C concentrations increased in all cases and reached a peak of 176 ± 71 μmol/L at 1–4 min, whereas the corresponding peripheral vein vitamin C concentrations were 35 ± 15 μmol/L (P < 0.0001). Mean adrenal vein vitamin C increased from 39 ± 15 μmol/L at 0 min, rose to 162 ± 101 μmol/L at 2 min, and returned to 55 ± 16 μmol/L at 15 min. Adrenal vein vitamin C release preceded the release of adrenal vein cortisol, which increased from 1923 ± 2806 nmol/L at 0 min to 27 191 ± 16 161 nmol/L at 15 min (P < 0.0001). Peripheral plasma cortisol increased from 250 ± 119 nmol/L at 0 min to 506 ± 189 nmol/L at 15 min (P < 0.0001).

Conclusions: Adrenocorticotrophic hormone stimulation increases adrenal vein but not peripheral vein vitamin C concentrations. These data are the first in humans showing that hormone-regulated vitamin C secretion occurs and that adrenal vitamin C paracrine secretion is part of the stress response. Tight control of peripheral vitamin C concentration is permissive of higher local concentrations that may have paracrine functions.

KEY WORDS Vitamin C, adrenal gland, stress response, cortisol, paracrine secretion

INTRODUCTION

The physiologic response to stress is coordinated by the pituitary gland, which secretes tropic hormones in response to central nervous system input from the hypothalamus. The essential adrenocorticotrophic hormone (ACTH) secreted by the pituitary gland stimulates adrenal glands to synthesize and secrete cortisol. In animals, ACTH also causes vitamin C loss from adrenals (1–3). Adrenal glands are rich in vitamin C, with concentrations as high as 10 mmol/L (4). For these reasons, vitamin C and stress in humans have long been associated, despite a lack of direct evidence for such a link. There are no human data, and animal evidence is inconsistent regarding utilization within adrenals or release from adrenals that could increase vitamin C concentrations in either local or systemic veins (4).

Humans, unlike most animals, cannot synthesize vitamin C and instead must obtain it from diet. Healthy humans consuming 200–300 mg vitamin C/d, an amount obtainable from foods such as fruit and vegetables in which the vitamin is abundant, maintain steady-state fasting plasma concentrations of 70 to 80 μmol/L (5, 6). Tightly controlled plasma vitamin C concentrations are exceeded transiently with oral doses of ≥1 g in amounts obtainable only from supplements and not from foods. Concentrations produced by supplement doses of ≥500 mg would not occur in nature (7). In tissues other than red blood cells, vitamin C intracellular concentrations are usually maintained in the millimolar range, in contrast to the micromolar range in plasma (8, 9). The observed tight control of vitamin C plasma and tissue concentrations is mediated by gastrointestinal absorption, cellular transport, and renal reabsorption and excretion. The especially tight control of plasma concentrations resulting from ingestion of vitamin C amounts found in foods (5–7) could facilitate paracrine actions of the vitamin, if local concentrations were higher. We hypothesized that the adrenal glands secrete vitamin C after simulated stress and that tight control of plasma vitamin C concentrations would permit intraadrenal vitamin C concentrations to be far higher than those in peripheral veins. To test this, we studied patients with hyperaldosteronism who underwent adrenal vein sampling for specific diagnosis. In these patients, we measured adrenal and peripheral vein vitamin C and cortisol concentrations after ACTH administration.

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2 JL Doppman is deceased.

3 Presented in part at the Endocrine Society Annual Meeting, June 2004, in New Orleans, LA.

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SUBJECTS AND METHODS

Subjects
We studied 16 men and 10 women aged 52.3 ± 8.6 y (range: 32–68 y) who underwent bilateral adrenal vein sampling at the National Institutes of Health. Subjects had clinical and biochemical features of hyperaldosteronism and were referred for adrenal vein sampling to differentiate between aldosterone-secreting adrenal adenoma and hyperplasia as the cause of hyperaldosteronism.

All patients gave written informed consent. The study protocol was approved by the institutional review board of the National Institutes of Health.

Adrenal vein sampling
Adrenal vein sampling was performed in the morning after an overnight fast. Patients received 2 mg midazolam given intravenously at the beginning of the procedure. Two peripheral venous cannulae were inserted, one for blood sampling and the other for drug infusions. Both adrenal veins were cannulated via the femoral vein, and cannulation was guided by digital subtraction angiography (10). Blood samples were drawn from each adrenal vein and the peripheral vein at time 0. A 250-μg bolus of ACTH was given intravenously and then 250 μg ACTH in 250 mL normal saline was infused intravenously at a rate of 200 mL/h. Blood samples were collected at 0, 2, 4, 6, 8, 10, and 15 min.

Assays
All samples were assayed for vitamin C and cortisol concentrations. The blood samples were kept on ice until sampling ended. Plasma was processed at 4 °C for vitamin C and cortisol analyses as described previously (5, 11). Briefly, 1–5 mL heparinized whole blood was centrifuged at 1000 × g for 10 min at 4 °C. Plasma (supernatant) was removed, diluted 1-in-5 with 90% methanol/water containing 1 mmol EDTA/L, and vigorously mixed by vortex for 10 s. Precipitated protein was removed by centrifugation at 25 000 × g for 20 min at 4 °C. Supernatants were stored at −80 °C until they were analyzed. For ascobic acid, all samples from the same patient were assayed together by using HPLC with coulometric electrochemical detection (12, 13). The intraassay and interassay CVs were <1% and <3%, respectively. Plasma cortisol was measured by using Immulite 2000 Cortisol Immunoassay. The intraassay and interassay CVs were 6% and 9%, respectively.

Statistical analysis
Results were compared by using paired t tests or repeated-measures analysis of variance (ANOVA) with Bonferroni’s post test when appropriate, and 2-tailed P values were calculated. Adrenal vein samples taken from the right and left adrenal glands were related because they were from the same patient. However, there were variations between the right and left values because of differences in catheter position, venous anatomy, or possibly other local factors. Because not all of the 47 available vitamin C and cortisol measurement pairs were statistically independent (they came from 26 subjects, most of whom contributed 2 measurement pairs), we used an adjusted calculation to compute the significance of the observed sample correlation. This calculation takes into account this clustering (observations are independent among clusters but may be dependent within a cluster) and uses a robust variance calculation (the Huber-White sandwich estimator of variance) in STATA statistical software (release 8.2; Stata Corporation, College Station, TX).

RESULTS
On adrenal vein catheterization, blood samples were successfully obtained from both adrenal glands in 21 patients and from one adrenal gland in 5 patients. Twenty-one patients had unilateral adrenal adenoma and 5 had bilateral adrenal hyperplasia. For this study, samples were assayed for vitamin C and cortisol. After ACTH stimulation, adrenal vein vitamin C concentrations increased in every adrenal vein sampled (Figure 1). The highest values, mean ± SD concentrations of 176 ± 71 μmol/L, were reached between 1 and 4 min, and these were significantly (P < 0.0001, paired t test) higher than corresponding peripheral plasma vitamin C concentrations (35 ± 15 μmol/L). In those patients in whom adrenal vein vitamin C concentration could be measured in only one adrenal gland, that single value was used in the calculation. In those in whom both adrenals were successfully sampled, the mean of the 2 adrenal vein vitamin C concentrations was used for statistical calculation, but all values are shown in the figure.

FIGURE 1. Vitamin C concentrations in the adrenal and peripheral veins in 26 patients with primary hyperaldosteronism. Under radiographic guidance, catheters were placed in both adrenal veins, and blood samples were taken after stimulation with adrenocorticotrophic hormone. Vitamin C concentrations in each of the adrenal (n = 47) and peripheral (n = 26) veins sampled are shown. In 5 patients, blood samples were obtained from only one adrenal vein because of unusual venous anatomy or technical difficulties with adrenal vein catheterization. In the adrenal veins, peak vitamin C concentrations (1 ± SD: 176 ± 71 μmol/L) were reached between 1 and 4 min, and these were significantly (P < 0.0001, paired t test) higher than corresponding peripheral plasma vitamin C concentrations (35 ± 15 μmol/L). In those patients in whom adrenal vein vitamin C concentration could be measured in only one adrenal gland, that single value was used in the calculation. In those in whom both adrenals were successfully sampled, the mean of the 2 adrenal vein vitamin C concentrations was used for statistical calculation, but all values are shown in the figure.
Adrenal vein cortisol concentrations increased from 1923 ± 2806 nmol/L at 0 min to 27 191 ± 16 161 nmol/L at 15 min. Peripheral plasma cortisol increased from 119 nmol/L at 0 min to 506 ± 189 nmol/L at 15 min (P < 0.0001, paired t test). Peripheral vein vitamin C concentrations showed a significant difference between the 3 slopes.

Increased from 39 ± 15 μmol/L at 0 min to 162 ± 101 μmol/L at 2 min and returned to 55 ± 16 μmol/L at 15 min (Figure 2). Bonferroni-adjusted adrenal vein vitamin C concentrations at 2 min were significantly greater than those at 0, 6, 8, 10, and 15 min. Peripheral vein vitamin C concentrations showed a significant change (P = 0.002 by repeated-measures ANOVA with Huynh-Feldt correction), but the direction of change was inconsistent and its magnitude was small (range: 32–37 μmol/L). Adrenal vein cortisol concentrations increased from 1923 ± 2806 nmol/L at 0 min to 27 191 ± 16 161 nmol/L at 15 min (P < 0.0001). Peripheral plasma cortisol increased from 250 ± 119 nmol/L at 0 min to 506 ± 189 nmol/L at 15 min (P < 0.0001) (Figure 3).

Because adrenal vein catheterization is an invasive procedure with a small risk of serious complications, vitamin C secretion in healthy persons was not studied. To address whether normal adrenal glands secrete vitamin C and, if so, to determine whether it differs from abnormal adrenal glands with respect to vitamin C secretion, we compared ascorbic acid secretion from the 21 patients with unilateral adrenal adenomas with the contralateral normal adrenals in the same patients (Figure 3). The data show that ascorbic acid secretion did not differ significantly according to whether adrenal adenoma was present or not. Peak adrenal vein vitamin C and cortisol concentrations were strongly correlated in all adrenals (r² = 0.35, P < 0.001; Figure 4). The correlations between peak adrenal vein vitamin C and cortisol for normal adrenal glands, for adrenal glands with adenoma, and for hyperplastic adrenal glands are shown. Analysis of covariance with interaction to test whether the 3 slopes were equal found no significant difference between the 3 slopes.

DISCUSSION

These data are the first description in any species of simultaneous adrenal vein and peripheral vitamin C concentrations after ACTH stimulation and the first to indicate that the putative function of secreted vitamin C must be local rather than systemic. After ACTH stimulation, peak adrenal vitamin C concentrations (176 ± 71 μmol/L) were significantly (P < 0.0001) higher than matched peripheral vein vitamin C concentrations (35 ± 15

**FIGURE 2.** Mean (±SD) vitamin C and cortisol concentrations in the peripheral and adrenal veins of all patients studied (n = 26). Adrenal vitamin C concentrations were measured in the right and left adrenals in most but not all subjects. When adrenal vitamin C concentrations were available from only one side, values from that side were used for statistical calculations, but the mean of the right and left adrenal vein vitamin C concentrations was used when both were available. Repeated-measures ANOVA of adrenal vein vitamin C concentrations gave a Huynh-Feldt–corrected P value of < 0.0001. Mean adrenal vein vitamin C increased from 39 ± 15 μmol/L at 0 min to 162 ± 101 μmol/L at 2 min and returned to 55 ± 16 μmol/L at 15 min. Bonferroni-adjusted adrenal vein vitamin C concentrations at 2 min were significantly greater than those at 0, 6, 8, 10, and 15 min. The mean highest value was 4.1-fold the mean lowest value. Peripheral vein vitamin C concentrations were obtained for all patients. Repeated-measures ANOVA gave a Huynh-Feldt–corrected P value of 0.002 for peripheral vein vitamin C concentrations, but values varied by only 8–16%, and the direction of change was inconsistent. Because we studied these patients for only 15 min, whereas peripheral vein cortisol concentrations would continue to increase for much longer, we compared 0- and 15-min cortisol values. Adrenal vein cortisol increased from 1923 ± 2806 nmol/L at 0 min to 27 191 ± 16 161 nmol/L at 15 min (P < 0.0001 paired t test). Peripheral plasma cortisol increased from 250 ± 119 nmol/L at 0 min to 506 ± 189 nmol/L at 15 min (P < 0.0001, paired t test).

**FIGURE 3.** Mean (±SD) adrenal vein vitamin C concentrations for all patients (n = 21) on the side with the normal adrenal gland and the side with an adrenal adenoma. The area under the curve of adrenal vein vitamin C concentrations in these 2 groups did not differ significantly (P = 0.57, unpaired t test).

**FIGURE 4.** Correlation between the highest vitamin C and cortisol concentration reached in each of the sampled adrenal veins for all the adrenal glands sampled (—). Correlation between peak vitamin C and cortisol for each of the adrenal glands is also shown for normal adrenal glands (△), for adrenal glands with adenoma (●), and for hyperplastic adrenal glands (●). The relation between peak adrenal vein vitamin C and cortisol for normal adrenal glands (—), for adrenal glands harboring an adenoma (—), and for hyperplastic adrenal glands (—■—) are shown. ANCOVA with interaction to test whether the 3 slopes were equal showed no significant difference between the 3 slopes.
vitamin C concentrations do not exceed 70–80 μmol/L, this was not a sustained increase but rather a secretory peak, and the highest mean value at 2 min was significantly greater than the values at 0, 6, 8, 10, and 15 min. Such a peak did not occur in the peripheral vein and could have only a local action in the adrenal gland. Cortisol release was clearly preceded by vitamin C release, which was waning as cortisol release increased. Small variations seen in the peripheral vein vitamin C concentrations were inconsistent in direction and much smaller in magnitude than those that follow normal meals. Hence, these variations are unlikely to have any physiologic significance.

A rapid increase in adrenal vein but not peripheral vein vitamin C concentrations provides several novel insights. One insight is that, in humans, adrenal vitamin C secretion is an integral part of the stress response. The function of released vitamin C in the stress response is unknown, but it may include the quenching of oxidants released during steroidogenesis (14); nitric oxide vasodilation, which may increase cortisol delivery to the medulla, the vena cava, or both; or the modification of ACTH receptor sensitivity. In addition, part of medullary blood originates in the adrenal cortex and is enriched with cortisol and vitamin C secreted by the adrenal cortex. Vitamin C is a cofactor necessary for the synthesis of norepinephrine localized to the adrenal medulla, whereas cortisol increases epinephrine biosynthesis from norepinephrine in adrenal medulla by up-regulating phenylethanolamine-N-methyltransferase. The local medullary vitamin C concentrations resulting from ACTH-induced vitamin C release ensure that norepinephrine synthesis always proceeds at maximum velocity \( V_{\text{max}} \) (16, 17). Because norepinephrine is the substrate for epinephrine synthesis, and because local cortisol may up-regulate phenylethanolamine-N-methyltransferase, the combined effects of vitamin C– and cortisol-enriched blood from adrenal cortex could also ensure that epinephrine synthesis proceeds at \( V_{\text{max}} \) in the adrenal medulla (4, 18).

Another insight, supported by the new data presented here, is the concept that one purpose of tight control of plasma vitamin C concentrations is to allow much higher local intraadrenal concentrations to occur transiently. When vitamin C is obtained from foods, despite varied dietary intakes, fasting steady-state plasma vitamin C concentrations do not exceed 70–80 μmol/L in humans (5–7). In another insight, as shown here, the function of released vitamin C must be local, within adrenals, rather than systemic. Furthermore, because of blood sampling limitations, the measured concentrations very likely underestimate true intraadrenal concentrations. Sampled blood reflects the dilution of adrenal vein outflow that is due to catheter placement. Ascorbate released within adrenal is diluted in an increasing venous blood volume before reaching the catheter. Thus, tight control of peripheral plasma vitamin C concentrations may permit the occurrence of much higher concentrations of locally released vitamin, and such concentrations may have special functions. As a corollary and as another novel insight, we show, for the first time in humans, hormone-stimulated secretion of any vitamin, not just vitamin C. These data indicate that a substance that is an essential nutrient may also have unanticipated paracrine or local hormone-like properties.

Adrenal vein catheterization is a technically challenging procedure, further complicated by variations in adrenal vein drainage. It is often unclear whether low cortisol concentrations in the adrenal vein blood result from catheter displacement or some other reason. Measurement of adrenal vein vitamin C concentration is useful as an additional measure of catheter placement and is so used at our institution now. Efforts are underway to develop a rapid vitamin C assay that will give an answer while the patient is still on the table—ie, before catheterization has ended.

If adrenal vitamin C secretion has physiologic consequences, consideration should be given to vitamin C intake above that possible from foods alone. Vitamin C supplements of 1 g, taken twice daily as a supplement, can produce transient peak plasma concentrations of \( \approx 140 \) μmol/L. Higher doses taken more frequently—eg, every 4–6 h—may produce transient peak plasma concentrations approaching 200 μmol/L, and the average concentrations would be only slightly lower (7) These concentrations are possible only from either oral supplements or intravenous injection; would be expected to be distributed uniformly in plasma, including adrenal veins; and simulate some concentrations measured in adrenal vein samples in this study. However, these concentrations do not reflect the higher intraadrenal concentrations expected with ACTH-induced vitamin C release. It is not known whether such concentrations produced by supplements will have inadvertent paracrine signaling consequences. Finally, we cannot determine from the data presented here whether vitamin C secretion occurs during episodic ACTH secretion by the pituitary gland.

We thank Mark E. Ruddel for performing the cortisol analyses, Anthony Lafferty for contributions to patient care, and Robert Wesley for statistical advice.

The authors’ responsibilities are as follows—ILD, JG, and ML: study concept and design; SIP, JLD, RC, WY, DAP, and ML: data collection and analysis and interpretation of results; SIP and ML: writing of the manuscript; and all authors (except JLD, who is deceased) reviewed the final manuscript. The funding source had no role in study design, collection, analysis and interpretation of data, or in writing or in submitting the paper for publication. None of the authors had a personal or financial conflict of interest.

REFERENCES
Risk factors for low serum 25-hydroxyvitamin D concentrations in otherwise healthy children and adolescents1–3

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ABSTRACT
Background: Serum 25-hydroxyvitamin D [25(OH)D] concentrations serve as a biomarker for vitamin D stores. Prior studies have not examined the risk factors for low vitamin D concentrations in a multicohort sample of US youth across a broad age range.
Objective: The objective was to determine the prevalence of and factors associated with low concentrations of 25(OH)D in children and adolescents.
Design: Serum 25(OH)D concentrations were measured in 382 healthy children aged 6–21 y living in the northeastern United States. Dietary and supplemental vitamin D intake was assessed by interview. Fat and lean mass were assessed by dual-energy x-ray absorptiometry. Multivariable ordinal logistic regression was used to determine factors associated with decreased concentrations of 25(OH)D.
Results: The median concentration of 25(OH)D was 28 ng/mL (interquartile range: 19–35 ng/mL), and 55% of subjects had 25(OH)D concentrations <30 ng/mL. 25(OH)D concentrations were inversely correlated with parathyroid hormone concentrations (Spearman’s r = −0.31, P < 0.001) but were not significantly correlated with 1,25-dihydroxyvitamin D concentrations. In the multivariable model, older age (P < 0.001), black race [odds ratio (OR): 14.2; 95% CI: 2.29, 5.50], and total daily vitamin D intake <200 IU (OR: 1.58; 95% CI: 1.02, 2.46) were associated with low vitamin D concentrations. Fat and lean mass were not independently associated with vitamin D status in this healthy-weight sample.
Conclusion: Low serum 25(OH)D concentrations are prevalent in otherwise healthy children and adolescents in the northeastern United States and are related to low vitamin D intake, race, and season. Am J Clin Nutr 2007;86:150–8.

KEY WORDS Vitamin D, 25-hydroxyvitamin D, children, adolescents, 1,25-dihydroxyvitamin D, parathyroid hormone

INTRODUCTION
It is well recognized that adequate stores of vitamin D are crucial for musculoskeletal health (1, 2). The best indicator of vitamin D stores is the serum concentration of calcidiol, or 25-hydroxyvitamin D [25(OH)D] (3, 4). When circulating 25(OH)D concentrations are inadequate, a state known as hypovitaminosis D, intestinal calcium absorption and bone mineralization are impaired. More severe deficits in 25(OH)D lead to clinical myopathy, osteomalacia in adults, and rickets in children (5). A recent randomized clinical trial of vitamin D supplementation in healthy school-aged girls in Lebanon showed significant beneficial effects on lean mass and bone mineral content, especially during the premenarchal period (6). In Finnish military recruits, stress fractures were associated with low vitamin D status (7). In addition to its musculoskeletal effects, vitamin D is important for immune function, and hypovitaminosis D may contribute to varied diseases, such as hypertension, cancer, multiple sclerosis, and type 1 diabetes (2).

Hypovitaminosis D remains an underrecognized problem in the general population and is poorly defined in children. Recent studies showed inadequate circulating 25(OH)D concentrations in adult medical inpatients (8), postmenopausal women (9), and free-living adults (10). In the pediatric population, several studies documented low serum vitamin D concentrations in adolescents living in Boston, Cleveland, and Maine (11–13), in infants and toddlers (14) in Alaska, and in children of primary school age in Lebanon (15). Of substantial concern, given the current obesity epidemic, is that obesity in children was also shown to be associated with decreased 25(OH)D concentrations (11, 16); however, these prior studies determined obesity by using body mass index (BMI; in kg/m2) rather than a more direct estimate of body fat mass. In addition, prior studies did not examine the relations between vitamin D status, race, body composition, and dietary intake in children across a broad age range. The aims of this study were to determine (1) the prevalence of serum 25(OH)D concentrations <30 ng/mL—a recognized indicator of hypovitaminosis D in adults (17) and of more severe deficits of 25(OH)D in children and adolescents—and (2) the factors associated with reduced 25(OH)D concentrations.

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SUBJECTS AND METHODS

Study design and participants

A cross-sectional study of skeletal development in healthy children aged 6-21 y from the Philadelphia, PA, area (at latitude 40°N) was conducted, and blood samples were obtained in a subset of the participants. Study participants were recruited through newspaper advertisements, mailings, and fliers to primary care centers and pediatric practices affiliated with The Children’s Hospital of Philadelphia and the surrounding community. For inclusion, children had to have a reported height, weight, and BMI (at the time the appointment was scheduled) within the 5th to 95th percentiles (18). Children were excluded if they used any medications or had any chronic medical conditions that might affect growth, body composition, dietary intake, or physical activity.

The protocol was approved by the Committee for the Protection of Human Subjects. For study participants <18 y of age, written informed consent was obtained from a parent or guardian and verbal assent from the subject; written informed consent was obtained from participants ≥18 y of age.

Vitamin D metabolites and related measures

A nonfasting blood sample was drawn between 0800 and 1700 to determine serum concentrations of 25(OH)D, 1,25(OH)2D, parathyroid hormone (PTH), and bone-specific alkaline phosphatase (BSAP). The serum was stored in aliquots at −70 °C and shipped in batches for analysis (Quest Diagnostics’ Nichols Institute, San Juan Capistrano, CA). Serum 25(OH)D was analyzed by 125I-labeled radioimmunoassay with a commercially available test kit (DiaSorin, Stillwater, MN) (19). The DiaSorin primary antibody showed equal reactivity with 25(OH)D2 and 25(OH)D3 and excellent correlation with HPLC, the gold standard method (20). The intra- and interassay CVs were 2.2% and 8.6%, respectively. The 1,25(OH)2D concentrations were determined by a radioreceptor assay that uses solid-phase extraction followed by competitive binding with calf thymus vitamin D receptor (21); the intra- and interassay CVs were 7-11% and 12-15%, respectively. Intact PTH concentrations were measured with the Nichols chemiluminescence assay (interassay CV: 7-9%), and serum BSAP was measured with a specific 2-site immunoradiometric assay (interassay CV: 8.5%).

For purposes of the analysis, we defined hypovitaminosis D as 25(OH)D concentrations <30 ng/mL, because this is a recognized cutoff for healthy vitamin D concentrations (5, 17). Study subjects were further categorized into 4 vitamin D status groups on the basis of 25(OH)D concentrations (<10, 10 to <20, 20 to <30, and ≥30 ng/mL). These cutoffs were used in a previously published study (22). In addition, the cutoffs of 20 and 30 ng/mL ensured that there were adequate numbers of study subjects in each category of vitamin D status (<20, 20 to <30, and ≥30 ng/mL) for the multivariable ordinal logistic regression analysis.

The reference ranges reported by the Nichols laboratory are as follows: for intact PTH the normal ranges are 9–59 pg/mL for ages 6-9 y, 11–74 pg/mL for ages 10-13 y, 9–69 pg/mL for ages 14-17 y, and 10–65 pg/mL for ages >17 y. For 1,25(OH)2D the reference ranges are 27–71 pg/mL for ages 3-17 y and 16–60 pg/mL for ages >17 y. For BSAP, the reference ranges for female children and adolescents are 41.0-134.6 μg/L for ages 6-9 y, 24.2-154.2 μg/L for ages 10-13 y, 10.5-75.2 μg/L for ages 14-17 y, and 3.9-15.1 μg/L for ages >17 y. For male children and adolescents, the BSAP reference ranges are 41.0-134.6 μg/L for ages 6-9 y, 43.8-177.4 μg/L for ages 10-13 y, 13.7-128.0 μg/L for ages 14-17 y, and 5.9-22.9 μg/L for ages >17 y.

Growth, puberty status, and body composition

The height and weight of the subjects (in lightweight clothing and with shoes and adornments removed) were assessed with standard techniques (23). Age- and sex-specific SD scores (z scores) for BMI, height, and weight were calculated by using national reference standards (18). Sexual maturation was determined with a self-assessment pictorial questionnaire (24) that illustrated the 5 stages of development as described by Tanner (25). The self-assessment examination was carried out in a private room equipped with a mirror for self-examination, and assistance from the parent or guardian was available if needed. This self-assessment questionnaire was validated in our laboratory for children with Crohn disease (26).

Dietary intake

Dietary intakes of calcium and vitamin D were assessed via three 24-h recall interviews conducted by a research dietitian within 3 wk of the study visit. The first interview was conducted in person during the study visit, and the other 2 interviews were conducted by telephone. Depending on the age of the child, the parent or guardian and the child were interviewed with the use of food models, portion booklets, or serving containers to assist in estimating serving size. Nutrient analysis was performed with the Minnesota Nutrition Data System (University of Minnesota, Minneapolis, MN). For the subjects reporting the use of dietary supplements, the calcium and vitamin D contents from supplement sources were recorded during the study interview and, when necessary, confirmed by telephone after the study visit. Dietary and supplemental vitamin D and calcium intakes were compared with the Adequate Intakes as described in the Dietary Reference Intakes for these micronutrients (27) to calculate a percentage of the Adequate Intake [(dietary intake/Adequate Intake) × 100]. Of note, the Adequate Intake of vitamin D is 200 IU/d and of calcium is 800 mg/d in children aged 4-8 y, 1300 mg/d in children aged 9-18 y, and 1000 mg/d in adolescents and young adults aged >18 y.

Body composition

Fat mass and lean body mass were determined by dual-energy X-ray absorptiometry (DXA). Whole-body DXA scans (software version 12.3; Hologic Discovery, Bedford, MA) were obtained in the array mode following standard positioning techniques. Children wore hospital scrubs to eliminate clothing artifacts. In our laboratory, the long-term in vitro CV for bone mineral density is <0.6%, and the in vivo CV is <1% (28). One investigator (BSZ) reviewed all scans to determine acceptability. The total body fat mass, excluding the head, was used in the analyses.

Fat mass is known to show age- and sex-related trends and to differ by ethnicity (blacks compared with nonblacks) (29). As has been suggested for adults and children, height is the appropriate measure for determining relative fatness (30, 31). In the absence of national reference data for total body fat measures, we used the method of Altman (32) to generate age-, sex-, and ethnicity-specific z scores for fat mass relative to height and for
lean body mass relative to height on the basis of the distribution within the sample.

**Demographic characteristics**

Race, ethnicity, family income, and educational level were determined with a structured interview questionnaire. We used the race and ethnicity categories established by the Census Bureau. The maximum educational level attained by the primary caretaker was categorized as high school or less, any college or technical school, completed college or technical school, any postcollege education, or unknown. Annual household income was elicited in $10,000 increments and categorized as $30,000–$60,000, $60,000–$99,000, and ≥$100,000.

**Statistical analyses**

All analyses were performed with STATA statistical software (versions 7.0 and 9.0; Stata Corp, College Station, TX). A 2-sided P value of 0.05 was the criterion for statistical significance.

Continuous variables are expressed as means ± SDs or as medians with total and interquartile (25th–75th percentiles) ranges. Categorical variables are expressed as proportions. The relations between 1,25(OH)₂D, BSAP, and 25(OH)D concentrations were assessed with Spearman’s correlation coefficients.

To determine characteristics associated with vitamin D status, a multivariable ordinal logistic regression model was tested with the use of the ologit procedure in STATA to fit proportional odds models (33). Ordinal logistic regression [which considers cutoff values for 25(OH)D] was applied instead of linear regression [which considers actual 25(OH)D concentrations] for several reasons. First, the assumption of linearity for linear regression was not plausible for 25(OH)D concentrations; eg, we did not anticipate that the clinical significance of a 10-point difference in vitamin D concentrations would be the same for 50 compared with 60 ng/mL as for 5 compared with 15 ng/mL concentrations. In addition, the 25(OH)D concentrations violated the assumption of normality for linear regression. We therefore chose to represent 25(OH)D concentrations by using cutoff values that were used in prior studies.

Subjects were grouped into 3 ordinal categories of vitamin D status on the basis of their serum 25(OH)D concentrations: 0 to <20, 20 to ≤30, and ≥30 ng/mL. To fit the multivariable model, an unadjusted ordinal logistic regression analysis was performed first to examine the association of vitamin D status with demographic, socioeconomic, growth, and dietary characteristics. Factors for which the unadjusted odds ratios (ORs) had an associated P value <0.20 were eligible for inclusion in the multivariable model. Second, we manually fitted a parsimonious multivariable model, using a backward, stepwise strategy, with P < 0.10 as the inclusion criterion. Finally, the goodness of fit of the proportional odds model was assessed by comparing the fitted proportional odds models with multinomial logit models (mlogit, STATA) that do not assume any ordering in the categories of vitamin D status, as suggested in the STATA manual (version 9.0; R, page 345). Multiplicative interaction terms were used to test for interactions between risk factors for hypovitaminosis D.

To assess whether the effect of risk factors was the same across vitamin D categories, 2 binary logistic regression models were constructed that separately compared each higher vitamin D category with the lowest category, as additional confirmation of the ordinal logistic regression models.

**RESULTS**

**Subject demographic and growth characteristics**

The study enrolled 638 subjects, and vitamin D data were available for 382 subjects. Vitamin D data were not available for all subjects because some of the subjects refused to give blood or an insufficient quantity of serum was obtained for analysis. The subjects for whom vitamin D data were unavailable were significantly younger than those for whom vitamin D data were available (11.3 and 12.4 y; P < 0.0001), but the 2 groups did not differ in other demographic (including racial), growth, nutritional, or dietary characteristics or by season of study visit.

The demographic and growth characteristics of the 382 study participants are listed in Table 1. The ages of the study participants ranged from 6.0 to 21.6 y. The racial (37% black) and ethnic (7% Hispanic) distribution was typical of the Philadelphia metropolitan area. Forty-four percent of the study visits occurred during the months of November through March (inclusive). The household income and highest level of caregiver education were distributed across a broad spectrum. All the stages of puberty were represented in the sample. The linear growth status was typical of children in the United States, as indicated by the T scores for height. Because of the study screening criteria, the prevalence of obesity was lower than that of the US population (34); 17 children (4.5%) had a BMI z score greater than the 95th percentile. The median total daily vitamin D intake, calculated as the sum of the dietary intake and supplemental intake of vitamin D, was 206 IU (interquartile range: 116–359 IU). Total calcium intake is shown in Table 2.

**Prevalence of hypovitaminosis D and other serum markers**

The median serum concentration of 25(OH)D was 28 ng/mL (interquartile range: 19–35 ng/mL). The 5th and 95th percentiles for the distribution of 25(OH)D concentrations were 10 and 50 ng/mL, respectively. The distribution of subjects across ranges of vitamin D concentrations is shown in Table 3 and by race and season in Figure 1. There were no significant differences in 25(OH)D concentrations between winter and spring and between summer and fall, and thus the 4 seasons were collapsed into 2 broader seasons of wintertime (November through March, inclusive) and summertime (April through October, inclusive). Fifty-five percent of subjects had 25(OH)D concentrations that were inadequate (<30 ng/mL). In children examined during wintertime, the overall prevalence of hypovitaminosis D was 68%; the prevalence was 51% in whites and 94% in blacks. The median intact PTH, available for 322 subjects, was 33 pg/mL (interquartile range: 24–45 pg/mL) and 4% of the subjects had PTH values above the age-specific reference ranges. PTH was shown to be inversely associated with 25(OH)D (Spearman’s r = −0.31, P < 0.001). The distribution of PTH relative to 25(OH)D concentrations is shown in Figure 2.

As shown in Table 1, 1,25(OH)₂D concentrations were available for 187 subjects: 1 had low concentrations, 67% had normal concentrations, and 33% had concentrations above the upper limit for age. BSAP concentrations were available for 223 subjects: 92% had concentrations in the normal range for age and sex. 25(OH)D concentrations were not correlated with 1,25(OH)₂D (Spearman’s r = −0.07, P = 0.36) or BSAP (Spearman’s r = −0.04, and P = 0.60).
### TABLE 1
Characteristics of the study sample (n = 382)\(^1\)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>12.6 (9.9–15.0)(^2)</td>
</tr>
<tr>
<td>6 to 9 y [n (%)]</td>
<td>80 (21)</td>
</tr>
<tr>
<td>9 to &lt;12 y [n (%)]</td>
<td>84 (22)</td>
</tr>
<tr>
<td>12 to &lt;15 y [n (%)]</td>
<td>122 (32)</td>
</tr>
<tr>
<td>(\geq 15) [n (%)]</td>
<td>96 (25)</td>
</tr>
<tr>
<td>Male [n (%)]</td>
<td>182 (48)</td>
</tr>
<tr>
<td>Race [n (%)]</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>206 (54)</td>
</tr>
<tr>
<td>Black</td>
<td>141 (37)</td>
</tr>
<tr>
<td>Other, unknown, or refused</td>
<td>35 (9)</td>
</tr>
<tr>
<td>Ethnicity [n (%)]</td>
<td></td>
</tr>
<tr>
<td>Not Hispanic</td>
<td>330 (86)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>27 (7)</td>
</tr>
<tr>
<td>Unknown</td>
<td>25 (7)</td>
</tr>
<tr>
<td>Caregiver annual income [n (%)]</td>
<td></td>
</tr>
<tr>
<td>&lt;530 000</td>
<td>91 (24)</td>
</tr>
<tr>
<td>$30 000–599 999</td>
<td>92 (24)</td>
</tr>
<tr>
<td>(\geq 100 000)</td>
<td>81 (21)</td>
</tr>
<tr>
<td>Unknown</td>
<td>38 (10)</td>
</tr>
<tr>
<td>Highest caregiver education level [n (%)]</td>
<td></td>
</tr>
<tr>
<td>High school or less</td>
<td>87 (23)</td>
</tr>
<tr>
<td>Some college or technical school</td>
<td>91 (24)</td>
</tr>
<tr>
<td>Graduated from college or technical school</td>
<td>109 (29)</td>
</tr>
<tr>
<td>Some postcollege education</td>
<td>93 (24)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (&lt;1)</td>
</tr>
<tr>
<td>Month of study visit [n (%)]</td>
<td></td>
</tr>
<tr>
<td>December–February</td>
<td>96 (25)</td>
</tr>
<tr>
<td>March–May</td>
<td>104 (27)</td>
</tr>
<tr>
<td>June–August</td>
<td>98 (26)</td>
</tr>
<tr>
<td>September–November</td>
<td>84 (22)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>47.9 (31.6–58.9)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>155.8 (137.2–164.7)</td>
</tr>
<tr>
<td>BMI (kg/m)</td>
<td>19.2 (16.9–21.9)</td>
</tr>
<tr>
<td>Weight (z) score(^3)</td>
<td>0.4 (–0.3–0.9)</td>
</tr>
<tr>
<td>Height (z) score(^3)</td>
<td>0.2 (–0.4–0.7)</td>
</tr>
<tr>
<td>BMI (z) score(^4)</td>
<td>0.4 (–0.3–1.0)</td>
</tr>
<tr>
<td>Lean mass by DXA (kg)</td>
<td>33.6 (23.2–41.8)</td>
</tr>
<tr>
<td>Fat mass by DXA (kg)</td>
<td></td>
</tr>
<tr>
<td>&lt;5 [n (%)]</td>
<td>89 (23)</td>
</tr>
<tr>
<td>5–9.9 [n (%)]</td>
<td>132 (35)</td>
</tr>
<tr>
<td>10–14.9 [n (%)]</td>
<td>93 (24)</td>
</tr>
<tr>
<td>(\geq 15) [n (%)]</td>
<td>68 (18)</td>
</tr>
<tr>
<td>Tanner stage [n (%)]</td>
<td></td>
</tr>
<tr>
<td>Stage 1</td>
<td>90 (24)</td>
</tr>
<tr>
<td>Stage 2</td>
<td>53 (14)</td>
</tr>
<tr>
<td>Stage 3</td>
<td>69 (18)</td>
</tr>
<tr>
<td>Stage 4</td>
<td>103 (27)</td>
</tr>
<tr>
<td>Stage 5</td>
<td>63 (17)</td>
</tr>
<tr>
<td>Unknown</td>
<td>4 (1)</td>
</tr>
<tr>
<td>Dietary energy intake (kcal/d)</td>
<td>1817 (1535–2178)</td>
</tr>
<tr>
<td>Dietary vitamin D intake (% of AI)(^4)</td>
<td>85 (52–121)</td>
</tr>
<tr>
<td>Supplemenal vitamin D intake (IU/d)</td>
<td></td>
</tr>
<tr>
<td>0 [n (%)]</td>
<td>296 (78)</td>
</tr>
<tr>
<td>25–200 [n (%)]</td>
<td>15 (4)</td>
</tr>
<tr>
<td>400 [n (%)]</td>
<td>69 (18)</td>
</tr>
<tr>
<td>800 [n (%)]</td>
<td>2 (&lt;1)</td>
</tr>
<tr>
<td>Total daily vitamin D intake (IU)</td>
<td></td>
</tr>
<tr>
<td>&lt; 200 [n (%)]</td>
<td>180 (47)</td>
</tr>
<tr>
<td>(\geq 200) [n (%)]</td>
<td>202 (53)</td>
</tr>
</tbody>
</table>

\(^1\)DXA, dual-energy X-ray absorptiometry; AI, adequate intake; PTH, parathyroid hormone; 1,25(OH)\(_2\)D, 1,25-dihydroxyvitamin D.

\(^2\)Median; interquartile range (25th–75th percentiles) in parentheses (all such values).

\(^3\)Data missing for 3 persons.

\(^4\)Data missing for 7 persons.

\(^5\)Age-specific reference ranges given in Subjects and Methods.

\(^6\)Data available for 187 persons.

\(^7\)Data available for 223 persons.

### TABLE 1 (Continued)
Characteristics of the study sample (n = 382)\(^1\)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact PTH (pg/mL)(^7)</td>
<td>33 (24–45)</td>
</tr>
<tr>
<td>Low [n (%)]</td>
<td>1 (&lt;1)</td>
</tr>
<tr>
<td>Normal [n (%)]</td>
<td>307 (95)</td>
</tr>
<tr>
<td>High [n (%)]</td>
<td>14 (4)</td>
</tr>
<tr>
<td>1,25(OH)(_2)D (pg/mL)(^5,6)</td>
<td>62 (51–75)</td>
</tr>
<tr>
<td>Low [n (%)]</td>
<td>1 (&lt;1)</td>
</tr>
<tr>
<td>Normal [n (%)]</td>
<td>125 (67)</td>
</tr>
<tr>
<td>High [n (%)]</td>
<td>61 (33)</td>
</tr>
<tr>
<td>Serum calcium (mg/dL)</td>
<td>9.7 (9.4–9.9)</td>
</tr>
<tr>
<td>Bone-specific alkaline phosphatase (ng/mL)(^5,7)</td>
<td>67 (36–92)</td>
</tr>
<tr>
<td>Low [n (%)]</td>
<td>6 (3)</td>
</tr>
<tr>
<td>Normal [n (%)]</td>
<td>205 (92)</td>
</tr>
<tr>
<td>High [n (%)]</td>
<td>12 (5)</td>
</tr>
</tbody>
</table>

\(^7\)Data available for 223 persons.

### Factors associated with 25(OH)D concentrations

In the unadjusted ordinal logistic regression models, nearly all of the candidate demographic, growth, and nutritional characteristics (described in Table 1) were associated with vitamin D status groups (Table 4). Low vitamin D status was more likely to occur in study participants who were older, were black, were members of households with lower annual incomes, were members of households with lower caregiver educational levels, were evaluated during winter months, had greater fat mass, and had higher BMI \(z\) scores. Low vitamin D status was also associated with lower dietary, supplemental, and total daily intake of vitamin D. Of the candidate variables, only male sex, height \(z\) score, and daily energy intake were not significantly associated with vitamin D nutritional status \((P > 0.20)\).

The interpretation of the ORs presented from the ordinal logistic models in Table 4 and Table 5 is similar to the interpretation of the ORs in the binary logistic regression. For example, the OR for black subjects (compared with nonblack subjects) is 11.4. This implies that, for black subjects, the odds of being in the <30 rather than the \(\geq 30\) ng/mL category are 11.4 times the odds for nonblack subjects, given the assumption that all other variables in the model are held constant. Likewise, for blacks, the odds of being in the <20 rather than the \(\geq 20\) ng/mL category are 11.4

### TABLE 2
Total calcium intake by age group\(^1\)

<table>
<thead>
<tr>
<th>Age group</th>
<th>(\bar{x} \pm SD)</th>
<th>Median</th>
<th>Interquartile range(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;9 y ((n = 76))</td>
<td>766 ± 262</td>
<td>742</td>
<td>587–921</td>
</tr>
<tr>
<td>9–12 y ((n = 80))</td>
<td>812 ± 330</td>
<td>788</td>
<td>533–1030</td>
</tr>
<tr>
<td>12–15 y ((n = 123))</td>
<td>903 ± 494</td>
<td>839</td>
<td>547–1135</td>
</tr>
<tr>
<td>(\geq 15) y ((n = 94))</td>
<td>876 ± 485</td>
<td>761</td>
<td>520–1103</td>
</tr>
</tbody>
</table>

\(^1\)Total calcium intake is dietary calcium plus calcium in supplements.

\(^2\)25th–75th percentiles.
times the odds for nonblack subjects, given the assumption that all other variables in the model are held constant. The assumption that the odds for comparison of the >30 with the ≤30 ng/mL category are the same as for comparison of the >20 with the ≤20 ng/mL category, known as the proportional odds assumption, was tested and confirmed.

In the multivariable ordinal logistic regression model in which the independent variables are simultaneously adjusted for the other variables in the model, only older age, black race, a study evaluation during winter, and low total daily vitamin D intake were independently associated with decreased vitamin D status (Table 5). Fat mass, lean body mass, caregiver annual income, and other demographic, growth, and nutritional characteristics were not independently associated with vitamin D status. Multiplicative interaction terms between total daily vitamin D intake and a study evaluation during winter ($P = 0.11$) and between race and a study evaluation during winter ($P = 0.79$) were not statistically significant. That is, the associations between vitamin D status and race and between vitamin D status and vitamin D intake did not vary according to season.

**DISCUSSION**

Numerous adult studies examined the relations between serum concentrations of 25(OH)D and varied health outcomes and concluded that optimal serum concentrations are $>30$ ng/mL (17). In this cross-sectional study of otherwise healthy children and adolescents aged 6-21 y, more than one-half of the children had low vitamin D concentrations [25(OH)D $<30$ ng/mL], and the prevalence was $>90\%$ in black subjects during the winter months. In addition, a substantial proportion (19\%) of blacks assessed in the winter months had vitamin D concentrations $<10$ ng/mL, a concentration associated with clinical myopathy, osteomalacia, and rickets (5). In the unadjusted ordinal logistic regression models, numerous factors were associated with decreased vitamin D status. In the adjusted regression model, however, only older age, black race, study evaluation during winter months, and lower total intake of vitamin D were independently associated with decreased vitamin D status. The associations of low vitamin D

![Figure 1](image1.png) 25-Hydroxyvitamin D [25(OH)D] concentrations by race and season were not normally distributed. A 2-factor ANOVA showed significant differences by race ($P < 0.001$) and season ($P < 0.001$) but no significant interaction between race and season.

**TABLE 3**

Distribution of serum concentrations of 25-hydroxyvitamin D [25(OH)D]

<table>
<thead>
<tr>
<th>25(OH)D Value</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10 ng/mL</td>
<td>18 (5)</td>
</tr>
<tr>
<td>10 to &lt;20 ng/mL</td>
<td>79 (21)</td>
</tr>
<tr>
<td>20 to &lt;30 ng/mL</td>
<td>112 (29)</td>
</tr>
<tr>
<td>≥30 ng/mL</td>
<td>173 (45)</td>
</tr>
</tbody>
</table>
concentrations with black race and winter season were expected, given the findings from prior studies (11, 22, 35).

In adults, functional measures [net calcium absorption efficiency (36), normal serum PTH concentrations (37, 38), and fracture risk (39)] have been used to define vitamin D adequacy [serum 25(OH)D $>30$ ng/mL (40)]. Our data show that individuals with elevated PTH concentrations have serum 25(OH)D concentrations approaching 40 ng/mL. Further studies are needed to determine the serum 25(OH)D concentration that indicates vitamin D adequacy for children.

The inverse association of vitamin D status with age during childhood and adolescence has not been well described. Prior studies examined children within a narrow age range, such as adolescence (11–13) or infancy and toddlerhood (14), which limited the ability of these studies to determine whether vitamin D status varied with age. One recent study in girls aged 4-8 y reported an inverse relation between age and 25(OH)D concentrations in blacks but not in whites (35). Previously, our group noted lower vitamin D status with increasing age in children with steroid-sensitive nephrotic syndrome and in the healthy comparison group (22). The age-related decrease in vitamin D status was not attributable to decreased vitamin D intake, because we accounted for vitamin D intake in our multivariable model. Age-related decreases in physical activity (41) likely have an effect on outdoor activities and sun exposure, which represents a plausible explanation for the poorer vitamin D status of older children. Future studies should prospectively measure sun exposure and outdoor activities.

In our multivariable model, lean mass and fat mass adjusted for height were not associated with vitamin D status. Of note, a reported BMI greater than the 95th percentile at the time of the screening phone call was an exclusion criterion, and only 4% of the sample had a BMI in the overweight range. Prior studies in adults reported an association between hypovitaminosis D and obesity (42–44), in which fat mass was measured by whole-body DXA (42, 44) or bioelectrical impedance analysis (43). Obesity has also been shown to be associated with decreased 25(OH)D concentrations in children (11, 16) on the basis of a BMI-based categorization of obesity rather than a direct estimate of fat mass. Other pediatric studies found no association between BMI and 25(OH)D (35). Our lack of association between fat mass and vitamin D concentrations may have been due to the appropriate adjustment for the confounding effects of age, which was significant in our multivariable model. Prior reported associations between fat mass and hypovitaminosis D may have been confounded by the unmeasured effects of sunlight exposure during outdoor activity, because overweight subjects may have less exposure to sunlight and hence have hypovitaminosis D. The contribution of dietary quality and

![Figure 2](image-url)
### TABLE 4
Results of unadjusted ordinal logistic regression analyses for associations of subject characteristics with categories of 25-hydroxyvitamin D concentrations

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Ordinal odds ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (vs &lt;9 y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 to &lt;12 y</td>
<td>1.72 (0.94, 3.12)</td>
<td>0.0001</td>
</tr>
<tr>
<td>12 to &lt;15 y</td>
<td>3.30 (1.90, 5.71)</td>
<td></td>
</tr>
<tr>
<td>≥15 y</td>
<td>2.89 (1.62, 5.16)</td>
<td></td>
</tr>
<tr>
<td>Male (vs female)</td>
<td>1.28 (0.88, 1.86)</td>
<td>0.20</td>
</tr>
<tr>
<td>Black race (vs nonblack)</td>
<td>11.4 (7.18, 18.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ethnicity (vs not Hispanic)</td>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td>Hispanic</td>
<td>0.43 (0.20, 0.94)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>0.78 (0.37, 1.67)</td>
<td></td>
</tr>
<tr>
<td>Caregiver annual income (vs &lt;$30 000)</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$30 000–$60 000</td>
<td>0.65 (0.38, 1.10)</td>
<td></td>
</tr>
<tr>
<td>$60 000–$99 999</td>
<td>0.29 (0.16, 0.52)</td>
<td></td>
</tr>
<tr>
<td>≥$100 000</td>
<td>0.17 (0.10, 0.31)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>0.47 (0.23, 0.96)</td>
<td></td>
</tr>
<tr>
<td>Highest caregiver education level (vs high school or less)</td>
<td>0.81 (0.47, 1.40)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Some college or technical school</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Graduated from college or technical school</td>
<td>0.26 (0.15, 0.45)</td>
<td></td>
</tr>
<tr>
<td>Some postcollege education</td>
<td>0.34 (0.20, 0.60)</td>
<td></td>
</tr>
<tr>
<td>Month of study visit (vs December–February)</td>
<td>0.63 (0.38, 1.05)</td>
<td>0.002</td>
</tr>
<tr>
<td>March–May</td>
<td>0.37 (0.22, 0.63)</td>
<td></td>
</tr>
<tr>
<td>September–November</td>
<td>0.47 (0.27, 0.80)</td>
<td></td>
</tr>
<tr>
<td>Study visit during November through March (vs during other months)</td>
<td>2.73 (1.85, 4.01)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>1.03 (1.02, 1.04)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>1.02 (1.01, 1.03)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>1.12 (1.06, 1.18)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Weight z score</td>
<td>1.25 (1.01, 1.56)</td>
<td>0.04</td>
</tr>
<tr>
<td>Height z score</td>
<td>1.02 (0.82, 1.26)</td>
<td>0.88</td>
</tr>
<tr>
<td>BMI z score</td>
<td>1.23 (1.00, 1.50)</td>
<td>0.05</td>
</tr>
<tr>
<td>Fat mass by DXA (vs &lt;5 kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5–9.9 kg</td>
<td>1.84 (1.09, 3.10)</td>
<td></td>
</tr>
<tr>
<td>10–14.9 kg</td>
<td>1.90 (1.08, 3.34)</td>
<td></td>
</tr>
<tr>
<td>≥15 kg</td>
<td>2.85 (1.57, 5.16)</td>
<td></td>
</tr>
<tr>
<td>Fat mass-for-height z score</td>
<td>1.12 (0.93, 1.35)</td>
<td>0.24</td>
</tr>
<tr>
<td>Lean mass-for-height percentile by DXA (vs &lt;25th percentile)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25th–50th</td>
<td>0.69 (0.40, 1.19)</td>
<td></td>
</tr>
<tr>
<td>50th–75th</td>
<td>0.75 (0.44, 1.28)</td>
<td></td>
</tr>
<tr>
<td>&gt;75th</td>
<td>0.98 (0.59, 1.65)</td>
<td></td>
</tr>
<tr>
<td>Tanner stage (vs stage 1)</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Stage 2</td>
<td>2.18 (1.13, 4.21)</td>
<td></td>
</tr>
<tr>
<td>Stage 3</td>
<td>3.80 (2.03, 7.13)</td>
<td></td>
</tr>
<tr>
<td>Stage 4</td>
<td>3.58 (2.04, 6.28)</td>
<td></td>
</tr>
<tr>
<td>Stage 5</td>
<td>3.56 (1.91, 6.63)</td>
<td></td>
</tr>
<tr>
<td>Dietary energy intake (kcal/d)</td>
<td>1.00 (1.00, 1.00)</td>
<td></td>
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<tr>
<td>Dietary vitamin D intake (% of AI) (vs 0 to &lt;50%)</td>
<td></td>
<td>0.92</td>
</tr>
<tr>
<td>50 to &lt;75</td>
<td>0.71 (0.39, 1.27)</td>
<td></td>
</tr>
<tr>
<td>75 to &lt;100</td>
<td>0.29 (0.16, 0.55)</td>
<td></td>
</tr>
<tr>
<td>100 to &lt;125</td>
<td>0.44 (0.24, 0.81)</td>
<td></td>
</tr>
<tr>
<td>≥125</td>
<td>0.31 (0.17, 0.55)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>0.21 (0.04, 1.21)</td>
<td></td>
</tr>
<tr>
<td>Daily vitamin D supplementation (vs none)</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>25–200 IU</td>
<td>0.17 (0.05, 0.63)</td>
<td></td>
</tr>
<tr>
<td>≥400 IU</td>
<td>0.31 (0.18, 0.53)</td>
<td></td>
</tr>
<tr>
<td>Total daily vitamin D intake ≥200 IU (vs &lt;200 IU)</td>
<td>0.38 (0.26, 0.56)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

1 25-Hydroxyvitamin D concentrations were categorized into 3 ordinal categories of vitamin D status: 0 to <20, 20 to <30, and ≥30 ng/mL. The sample size for each variable is given in Table 1. AI, Adequate Intake.

2 The unadjusted ordinal odds ratios were calculated for each variable; the reference group for each variable is given in parentheses. For example, the odds ratio for the age group “12 to <15 y (vs <9 y)” is 3.30, which means that for those aged 12–15 y, the odds of being in the <30 vs the ≥30 ng/mL category are 3.3 times the odds of subjects aged <9 y; the odds are the same for the comparison of the <20 vs the ≥20 ng/mL category (on the basis of the confirmed proportional odds assumption).
supplement intake to the association of body fat and hypovitaminosis D also remains unknown.

Dietary vitamin D recommendations were established in 1997 (27) and were based on data available to estimate the intake needed to eliminate seasonal variations in PTH concentrations in adults. There were limited data such that an Adequate Intake, rather than a Recommended Dietary Allowance, was established. Many new studies have provided data for adults and children (45). The range of vitamin D required to optimize calcium absorption (36) is better understood, as is the range required to increase serum 25(OH)D concentrations (17, 46–49). In addition, the many non–bone health activities of vitamin D, such as those related to the risk of diabetes, obesity, and some cancers, are now recognized (2). Many children with chronic diseases have suboptimal vitamin D status (22, 50–52), and the current study provides evidence that this is cause for concern in otherwise healthy children. The findings reported here in this multi-ethnic sample of children and adolescents support the call for a review of the dietary recommendations for vitamin D (45) and underscore the importance of vitamin D intake.

This study has several notable strengths. We examined a large, relatively unselected sample of healthy children. Other studies described vitamin D status in more selected settings, such as in male students in a horseback-riding school (53) or female participants in a gymnastics study (35). Our study included children across a broad age range (6–21 y), to more thoroughly examine age trends. In addition, the sample included children from a broad range of socioeconomic and racial backgrounds and used multivariate statistical techniques to examine the relative contribution of these factors to vitamin D status. 25(OH)D concentrations were measured with the use of 125I-labeled radioimmunoassay, which has excellent results when compared with the gold standard liquid chromatography method (54, 55). Other pediatric studies (14, 56) used a chemiluminescence assay that may have overestimated total 25(OH)D and underestimated 25(OH)D2 (54, 57). We collected detailed information on intake of vitamin D from dietary and supplemental sources, which showed the important contribution of vitamin D intake to vitamin D status. Also, body fat was measured by DXA, rather than estimating body fat and obesity by using BMI. Finally, multivariable modeling with ordinal logistic regression was used in the analysis. Compared with traditional binary logistic regression, ordinal logistic regression retains the inherent ordinality of 25(OH)D concentrations and avoids arbitrary dichotomization. The categories of vitamin D status spanned a range of modest-to-severe deficits, and the analyses identified a similar effect of risk factors across vitamin D status categories.

This study has several limitations that should also be considered. The sample was conducted at a single site, so our results are not necessarily generalizable to all populations and geographic regions. However, our study sample was large and diverse, consisting of healthy children with few exclusion criteria, so our findings are likely generalizable to many other settings. Vitamin D data were only available for a subset of our full study sample, but selection bias was unlikely because the children who had vitamin D measurements were similar to the children who did not. Finally, sunlight exposure was not measured, but this diverse group of children was probably typical of other healthy-weight urban and suburban children in the mid-Atlantic states. Elucidation of the causes of low vitamin D concentrations in children will require studies that accurately measure dermal synthesis of vitamin D.

In conclusion, low vitamin D status was prevalent in otherwise healthy children in the mid-Atlantic United States. Low serum 25(OH)D concentrations were especially common during the winter months. In addition, older children, black children, and children with low total vitamin D intake were at risk of low circulating 25(OH)D concentrations. These children should be targeted for screening for hypovitaminosis D. Additional studies are needed to document the effects of screening for and treatment of hypovitaminosis D in otherwise healthy children.

We are deeply indebted to the children and their families who participated in this study and express our sincerest appreciation for their commitment to this research effort. Also, we thank the staff of the Nutrition and Growth Laboratory and the General Clinical Research Center at The Children’s Hospital of Philadelphia, the Joseph Stokes Research Institute, and Richard Reitz and the Nichols’ Institute of Quest Laboratories for their assistance with this project.

The authors’ responsibilities were as follows—all authors: writing of the manuscript; FLW and JS: design and interpretation of the data analysis; and BSZ, MBL, and VAS: overall study design, data collection, and data interpretation. None of the authors had any personal or financial conflicts of interest.

REFERENCES


<table>
<thead>
<tr>
<th>TABLE 5</th>
<th>Results of multivariable ordinal logistic regression analyses for categories of 25-hydroxyvitamin D concentrations1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>Ordinal odds ratio (95% CI)</td>
</tr>
<tr>
<td>Age (vs &lt;9 y)</td>
<td></td>
</tr>
<tr>
<td>9 to &lt;12 y</td>
<td>2.26 (1.13, 4.52)</td>
</tr>
<tr>
<td>12 to &lt;15 y</td>
<td>5.44 (2.85, 10.4)</td>
</tr>
<tr>
<td>≥15 y</td>
<td>5.01 (2.55, 9.83)</td>
</tr>
<tr>
<td>Black race (vs nonblack race)</td>
<td>14.2 (8.53, 23.5)</td>
</tr>
<tr>
<td>Study visit during winter (vs</td>
<td>3.55 (2.29, 5.50)</td>
</tr>
<tr>
<td>during nonwinter months)</td>
<td></td>
</tr>
<tr>
<td>Total daily vitamin D intake</td>
<td></td>
</tr>
<tr>
<td>&lt;200 IU (vs ≥200 IU)</td>
<td>1.58 (1.02, 2.46)</td>
</tr>
</tbody>
</table>

1 25-Hydroxyvitamin D concentrations were categorized into 3 ordinal categories of vitamin D status: 0 to <20, 20 to <30, and ≥30 ng/mL. The sample size for each variable is given in Table 1.

2 The adjusted ordinal odds ratios were calculated with all variables in the table included simultaneously in the model; the reference group for each variable is given in parentheses. For example, the odds ratio for “black race (vs. nonblack race)” is 14.2, which means that for black subjects, the odds of being in the <30 vs the ≥30 ng/mL category are 14.2 times the odds for nonblack subjects, assuming that all other variables in the model are held constant. The odds are the same for the comparison of the <20 vs the ≥20 ng/mL category (on the basis of confirmed proportional odds assumption).


Effect of folate oversupplementation on folate uptake by human intestinal and renal epithelial cells\(^1\text{-}\text{3}\)

Balasubramaniem Ashokkumar, Zainab M Mohammed, Nosratola D Vaziri, and Hamid M Said

**ABSTRACT**

**Background:** Folic acid plays an essential role in cellular metabolism. Its deficiency can lead to neural tube defects. However, optimization of body folate homeostasis can reduce the incidence of neural tube defects and may decrease the risk of Alzheimer and cardiovascular diseases and cancer. Hence, food fortification and intake of supplemental folate are widespread.

**Objective:** We examined the effects of long-term folate oversupplementation on the physiologic markers of intestinal and renal folate uptake processes.

**Design:** Human-derived intestinal Caco-2 and renal HK-2 epithelial cells were maintained (5 generations) in a growth medium over-supplemented (100 \(\mu\text{mol folic acid/L}\)) or maintained under sufficient conditions (0.25 and 9 \(\mu\text{mol folic acid/L}\)).

**Results:** Carrier-mediated uptake of \(^3\text{H}-\text{folic acid (2 \(\mu\text{mol/L}\)) at buffer pH 5.5 (but not buffer pH 7.4) by Caco-2 and HK-2 cells maintained under the folate-over-supplemented condition was significantly \((P < 0.01)\) and specifically lower than in cells maintained under the folate-sufficient condition. This reduction in folic acid uptake was associated with a significant decrease in the protein and mRNA levels of the human reduced-folate carrier (hRFC) and a decrease in the activity of the hRFC promoter. It was also associated with a decrease in mRNA levels of the proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1) and folate receptor (FR).

**Conclusions:** Long-term oversupplementation with folate leads to a specific and significant down-regulation in intestinal and renal folate uptake, which is associated with a decrease in message levels of hRFC, PCFT/HCP1, and FR. This regulation appears to be mediated via a transcriptional mechanism, at least for the hRFC system.

**KEY WORDS** Folate oversupplementation, intestinal folate uptake, renal folate uptake, hRFC, human reduced-folate carrier, PCFT/HCP1, proton-coupled folate transporter/heme carrier protein 1, folate receptor

**INTRODUCTION**

Folates play a fundamental physiologic role in one-carbon metabolism and are essential for the synthesis of precursors of nucleic acids, metabolism of several amino acids (including homocysteine), and initiation of protein synthesis in the mitochondria (1–3). Thus, it is not surprising that deficiency of this micronutrient leads to a variety of clinical abnormalities that range from neural tube defects to megaloblastic anemia (1–3). In contrast to the adverse effects of folate deficiency, optimization of body folate homeostasis was shown to lead to a significant reduction in the incidence of neural tube defects (4, 5) and omphalocoele (6). Some epidemiologic studies suggested that benefit may accrue from folate supplements for cardiovascular diseases (7), Alzheimer disease (8), and certain types of cancer [eg, colorectal cancer (9, 10)]. However, blinded, randomized clinical trials are raising serious doubts about such supplement benefits in some cases such as cardiovascular disease (11). Because of the benefits presumed, both realistically for birth defects and suggested epidemiologically for other medical problems, a mandatory fortification of grain products with folic acid was instituted in many countries, including the United States. Also, all women of childbearing age are advised now to take a daily supplement of folic acid (400 \(\mu\text{g/d}\)). Thus, the intake of folic acid from fortified food (\(\approx 100–200 \mu\text{g/d}\)) together with the use of nutritional supplements [that provide an additional 400 \(\mu\text{g folic acid/standard multivitamin preparation (12)}\] and consumption of nutrition bars and drinks [which are often supplemented with 400 \(\mu\text{g folic acid/}200\text{g serving (12)}\] create a state of folate oversupplementation in a significant segment of the population (12). This practice is occurring with little knowledge of the potential safety and physiologic consequences of chronic intake of such high doses of folic acid. A few reports, however, have raised questions about the validity of such practices (13–15). In this study, we examined the effect of long-term oversupplementation with folic acid on the physiology of folate transport in the human intestinal and renal epithelial cells.

The intestine plays a central role in regulating body folate homeostasis because the vitamin cannot be synthesized in the body and must be obtained from exogenous sources. Similarly, the kidneys play a pivotal role in regulating body folate homeostasis by reabsorbing the filtered vitamin, thus preventing its losses in the urine. Intestinal absorption of folate occurs via a specialized, acidic pH–dependent carrier-mediated process that involves the reduced folate carrier (RFC) (16–18) as well as a...
MATERIALS AND METHODS

[^3H]-folic acid (specific activity: 20 Ci/mmol; radiochemical purity: 98%) was obtained from Moravek Biochemicals (Brea, CA). TRIzol reagent and Lipofectamine were purchased from Life Technologies (Rockville, MD). DNA oligonucleotide primers were from Sigma Genosys (The Woodlands, TX). Routine biochemicals, enzymes, fetal bovine serum, and cell culture reagents were all of molecular biology quality and were purchased from either Fisher Scientific (Tustin, CA) or Sigma (St Louis, MO).

Cell culture and uptake studies

The human-derived intestinal epithelial Caco-2 cells and the human-derived renal proximal tubular epithelial HK-2 cells (ATCC, Manassas, VA) were used in this investigation. These cells were chosen because they have proven to be excellent models in such physiologic investigations and to yield data similar to those found with native intestinal and renal epithelial cells (25, 26). Caco-2 cells, a human colon carcinoma cell line, differentiate spontaneously in the culture to become small intestinal villus-like absorptive cells (27–29). HK-2 cells are an immortalized proximal tubule epithelial cell line from normal adult human kidney. Cells were grown and subcultured in custom-made Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum (Hyclone, Logan, UT), glucose (10 mmol/L), 2-(N-morpholino)ethanesulfonic acid/L, pH 5.5]. Labeled and unlabeled folic acid (2 μmol/L) were added to the incubation medium oversupplemented with folate (100 μmol/L). Another group of cells was maintained in the presence of an intermediate folate concentration of 9 μmol/L. Folic acid uptake was performed with the use of well-washed confluent monolayers (3–4 d after confluence) of Caco-2 (passages between 10 and 16) and HK-2 (passages between 8 and 16) cells. Uptake was measured at 37 °C in Krebs-Ringer solution [133 mmol NaCl/L, 4.93 mmol KCl/L, 1.23 mmol MgSO4/L, 0.85 mmol CaCl2/L, 5 mmol glucose/L, 5 mmol buffer [133 mmol NaCl/L, 4.93 mmol KCl/L, 1.23 mmol mannitol/L, 5 mmol EGTA/L, and 12 mmol Tris–HCl/L as oversupplementation with folic acid on intestinal and renal folate uptake process, we used Caco-2 and HK-2 cells as models. wells with the use of a Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Semiquantitative reverse transcription–polymerase chain reaction analysis of hRFC, PCFT/HCP1, and FR mRNA in Caco-2 and HK-2 cells maintained in the presence of different concentrations of extracellular folate

Total RNA was prepared with the use of the TRIzol reagent from confluent Caco-2 and HK-2 cells that were maintained in growth media containing sufficient and oversupplemented concentrations of folate. Total RNA (3 μg) was reverse transcribed with oligo (dT) primers with the use of Superscript II (Life Technologies) following the manufacturer’s procedures. After the reverse transcription (RT), all samples were diluted with sterile water, and 2 different dilutions were used for each polymerase chain reaction (PCR) with primer pairs specific for the hRFC, PCFT/HCP1, FR (both α and β isoforms), and the housekeeping gene β-actin to accurately determine their level of expression in the Caco-2 and HK-2 cells. Gene-specific primers corresponding to the PCR targets were designed by using the specifications given by the vendors (Bio-Rad Laboratories) and shown in Table 1. The conditions for semiquantitative RT-PCR were 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min (33 cycles). The products were analyzed on 2% agarose gels, the images were captured with the use of an Eagle Eye II system (Stratagene, LA Jolla, CA), and the amplified RT-PCR products were normalized to amplified β-actin gene controls as described previously (17).

Western blot analysis of hRFC and FR in Caco-2 and HK-2 cells maintained in growth media with different folate concentrations

Western blot analysis was performed with the use of the membranous fractions of Caco-2 and HK-2 cells maintained in varying concentrations of folate. The membranous fractions were isolated by homogenizing the cells in a buffer containing 300 mmol mannitol/L, 5 mmol EGTA/L, and 12 mmol Tris–HCl/L as

TABLE 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’–3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hRFC</td>
<td>Forward: ATGTCGCCCTCACGCCCAGCAGGTT</td>
<td>1776</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCACGCTTCACACC</td>
<td></td>
</tr>
<tr>
<td>PCFT/HCP1</td>
<td>Forward: ATGCAGCTTCTGGTTTGGT</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGACCCCATAGAGCTGGAC</td>
<td></td>
</tr>
<tr>
<td>FR</td>
<td>Forward: CAAGGTCAGCACTACAGCGGAG</td>
<td>108</td>
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<td></td>
<td>Reverse: CATGGCTGCACATAGACCTGCT</td>
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<tr>
<td>β-Actin 1</td>
<td>Forward: CTCCTGCGTCTGGACCT</td>
<td>750</td>
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<tr>
<td></td>
<td>Reverse: TAATGTCGACGATTTCC</td>
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<tr>
<td>β-Actin 2</td>
<td>Forward: CTCCTGCGTCTGGACCT</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>Reverse: TAATGTCGACGATTTCC</td>
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</tr>
</tbody>
</table>
well as a cocktail of protease inhibitors (1 mmol phenylmethyl-
sulfonyl fluoride/L, 1 μg aprotinin/mL, and 0.5 μg leupeptin/
ml) (30). Protein (100 μg) samples were treated with Laemmli
sample buffer and resolved on an 8% sodium dodecyl sulfate-
polyacrylamide gel. After electrophoresis, the proteins were
electroblotted on Hybond electrochemiluminescence nitrocellu-
lose membrane (Amersham Pharmacia Biotech, Piscataway,
NJ), washed twice with phosphate-buffered saline (PBS)–TWEEN
20 for 10 min, and blocked with 5% dried milk in PBS-TWEEN 20.
Blots were probed with previously validated rabbit anti-hRFC
polyclonal antibodies (1:1000 in PBS-TWEEN 20) (30). For FR,
blots were probed with polyclonal antibodies generated against
FR epitope corresponding to amino acids 1–257 (Santa Cruz
Biotechnology, Santa Cruz, CA). Immunodetection was per-
formed with goat anti-rabbit immunoglobin G conjugated to
horseradish peroxidase (1:1000 in PBS-TWEEN 20) with the use
of an enhanced chemiluminescence detection system (Amer-
sham, Arlington Heights, IL). Specific bands were measured
with the use of the Eagle Eye II system (Stratagene). Western
blotting for β-actin was performed as loading control. Blots were
incubated with a 1:1000 dilution of a goat anti-β-actin antibody
(Santa Cruz Biotechnology) and developed as described here
earlier.

hRFC promoter activity: transfection and reporter
gene assay

A fusion construct of the full-length hRFC promoter B (pB) with
the luciferase reporter gene (hRFC-pB-luciferase) prepared
in pGL3-basic vector, kindly provided by Dr. Larry H Matherly
of the Wayne State University School of Medicine, Detroit, MI,
was used in this investigation (31). The hRFC-pB-luciferase
construct was transfected into Caco-2 and HK-2 cells as de-
scribed previously (30). Cells were cotransfected in 12-well
plates at ≈70–75% confluence with 2 μg of each test construct
and 100 ng of the Renilla transfection control plasmid Renilla
luciferase-thymidine kinase (pRL-TK; Promega, Madison, WI).
Transfection was performed with Lipofectamine reagent (Invitrogen,
San Diego, CA) according to the manufacturer’s in-
cstructions. Cells were then harvested at 3–4 d after transfection
(confluence), and Renilla-normalized firefly luciferase activity
was determined by using the Dual Luciferase Assay system (Pro-
mega). Firefly luciferase activity was normalized relative to the
Renilla luciferase activity in the same cell extracts. Data are
presented as means ± SEM of at least 3 independent experi-
ments and given as folds over pGL3-Basic expression, which
was set arbitrarily at 1.

Data presentation and statistical analysis

Transport data presented in this study are mean ± SEM of
multiple separate uptake determinations and are expressed in
pico moles or femto moles per milligram protein per 7 min. Data
were analyzed with the use of analysis of variance (http://
faculty.vassar.edu/lowry/anova1u.html), with statistical signifi-
cance set at 0.05. All semiquantitative RT-PCR and Western blot
analyses were performed on at least 3 separate occasions with
comparable results, and data presented are from representative
sets of experiments.

RESULTS

Effect of folate oversupplementation on intestinal
folate uptake

The results of the effect of maintaining human intestinal epi-
thelial Caco-2 cells in folate-oversupplemented growth medium
on the uptake of 2 μmol folic acid/L at incubation buffer pH 5.5
are shown in Figure 1. A folic acid concentration of 2 μmol/L
was used to minimize the contribution of the FR-mediated uptake
of folate (because Caco-2 cells, but not the normal intestinal
epithelial cells, express this receptor (32), the apparent dissoci-
ation constant (Kd) of the FR is ≈10 nM (20–24)]. As can be seen
from Figure 1, uptake of 1H-folic acid by cells maintained in
folate-oversupplemented medium is significantly (P < 0.01)
lower than uptake by cells maintained in folate-sufficient med-
ium (0.25 μmol/L) (with the uptake by cells maintained in the
presence of 9 μmol/L folic acid falling in between). Uptake of the
unrelated water-soluble vitamin biotin (7.9 nmol/L), however,
was similar under the different folate conditions (322.1 ± 21.7,
329.4 ± 13.5, and 325.0 ± 18.3 fmol · mg protein−1 · 7 min−1
for cells maintained in the presence of 0.25, 9, and 100 μmol
folate/L, respectively).

We also examined the effect of folate oversupplementation on
uptake of folic acid (2 μmol/L) at buffer pH 7.4. As reported
before (33), uptake of folic acid by control Caco-2 cells (those
maintained under folate-sufficient condition) was markedly
(P < 0.01) lower at buffer pH 7.4 than at buffer pH 5.5 (0.11 ±
0.01 and 1.63 ± 0.09 pmol · mg protein−1 · 7 min−1, respec-
atively). Uptake of folic acid was not affected by the different
folate concentrations in the growth medium at this pH (Figure 1).

Effect of maintaining Caco-2 cells in folate-
oversupplemented growth medium on hRFC steady state
mRNA and protein and on the hRFC promoter activity

The effect of growing Caco-2 cells in folate-oversupplemented
growth medium on expression of the hRFC and RNA isolated from cells
grown under folate-oversupplemented and -sufficient conditions

![Figure 1. Effect of folate oversupplementation on intestinal folate uptake](http://example.com/figure1.png)
The results show the level of the hRFC mRNA (normalized to β-actin mRNA) to be significantly (P < 0.01) lower in cells maintained under folate-oversupplemented condition than in cells maintained under folate-sufficient condition.

Data on the effect of folate oversupplementation on hRFC protein expression is shown in Figure 2B. Western analysis was performed with the use of specific polyclonal anti-hRFC antibodies as described in “Methods.” The results showed a
The PCFT/HCP1 mRNA (normalized to and specific primers for the hRFC (Table 1). The results showed RT-PCR was performed with the use of RNA isolated from cells growth medium on effect of maintaining Caco-2 cells in folate-oversupplemented intestinal epithelial cells (19). In this study we examined the mRNA of PCFT/HCP1 oversupplemented growth medium on the steady state expression of the hRFC, and the PCFT/HCP1 systems function in the nanomolar range (20–24), the FR functions in the micromolar range (16, 18, 19). In this study, we determined the effect of maintaining the HK-2 cells in folate-oversupplemented growth medium on the mRNA expressions of hRFC, the PCFT/ HCP1 systems, and FR. Semiquantitative RT-PCR was performed on RNA isolated from cells grown under folate-oversupplemented and -sufficient conditions using specific primers for hRFC, PCFT/HCP1, and FR (Table 1). The results (Figure 4, A, B, and C) showed the mRNA of the hRFC, PCFT/ HCP1, and FR (normalized to β-actin gene) to be significantly (P < 0.01 for all) lower in cells maintained in folate-oversupplemented growth medium than in cells maintained in folate-sufficient media.

Effect of maintaining HK-2 cells in folate-oversupplemented growth medium on hRFC steady state mRNA and protein and on hRFC promoter activity

The normal kidney expresses FR, RFC, and PCFT/HCP1 systems (19–24). The FR functions in the nanomolar range (20–24), whereas the hRFC and the PCFT/HCP1 systems function in the micromolar range (16, 18, 19). In this study, we determined the effect of maintaining the HK-2 cells in folate-oversupplemented growth medium on the mRNA expressions of hRFC, the PCFT/ HCP1 systems, and FR. Semiquantitative RT-PCR was performed on RNA isolated from cells grown under folate-oversupplemented and -sufficient conditions using specific primers for hRFC, PCFT/HCP1, and FR (Table 1). The results (Figure 4, A, B, and C) showed the mRNA of the hRFC, PCFT/ HCP1, and FR (normalized to β-actin gene) to be significantly (P < 0.01 for all) lower in cells maintained in folate-oversupplemented growth medium than in cells maintained in folate-sufficient media.

Effect of maintaining HK-2 cells in folate-oversupplemented growth medium on protein expressions of hRFC and FR was also examined by Western analysis with the use of specific polyclonal antibodies as described in “Methods.” The results showed a significant (P < 0.01) reduction in the levels of both proteins in cells maintained under folate-oversupplemented conditions than in cells maintained under folate-sufficient conditions (Figure 4, D and E).

DISCUSSION

Our aims in these investigations were to examine the effects of long-term oversupplementation with folic acid on human intestinal and renal folate uptake. The physiologic and nutritional importance of such investigations relate because chronic supplementation with high concentrations of folic acid is widespread in the general population and because no study has investigated the possible effect of such long-term practice on the physiologic markers of folate, including its intestinal and renal uptake processes. Therefore, the present study was designed to address these issues with the intestinal epithelial Caco-2 cells and the renal epithelial HK-2 cells as models. Both of these human-derived epithelial cell lines have been shown to be excellent in

**FIGURE 3.** Effect of folic acid oversupplementation on [3H]-folic acid uptake by renal HK-2 cells. Confluent monolayers of HK-2 cells maintained in growth medium oversupplemented (100 µmol/L) or sufficient (0.25 µmol/L) in folate (as well as in the presence of an intermediate folic acid concentration of 9 µmol/L) were incubated at 37 °C in Krebs-Ringer buffer [pH 5.5 ([ ]) and pH 7.4 ([ ]) in the presence of 2 µmol/L [3H]-folic acid. Uptake was determined after 7 min of incubation. Values are mean ± SEM; n = 3, *P < 0.05; **P < 0.01.

Figure 3 shows the effect of folic acid oversupplementation on the uptake of [3H]-folic acid by renal HK-2 cells. The uptake was determined after 7 min of incubation. The results showed a significant (P < 0.01) reduction in hRFC protein abundance in cells maintained under folate-oversupplemented condition than in cells maintained under folate-sufficient condition.

We also examined the effect of folate-oversupplemented growth medium on the hRFC promoter activity in Caco-2 cells. We focused on the hRFC promoter because it is a prominent promoter used by variety of tissues (31, 34) [in fact, this promoter drives the expression of a hRFC variant 1, which is the predominant hRFC variant expressed in the normal intestine (18)] and has been well characterized (35). The results (Figure 2C) showed the activity of the hRFC promoter to be significantly (P < 0.01) lower in cells maintained in folate-oversupplemented growth medium than in cells maintained in folate-sufficient growth medium.

**Effect of maintaining Caco-2 cells in folate-oversupplemented growth medium on the steady state mRNA of PCFT/HCP1**

The PCFT/HCP1 is a recently identified folate transporter that is believed to play a role in normal folate uptake by human intestinal epithelial cells (19). In this study we examined the effect of maintaining Caco-2 cells in folate-oversupplemented growth medium on hRFC mRNA expression. Semiquantitative RT-PCR was performed with the use of RNA isolated from cells grown under folate-oversupplemented and -sufficient conditions and specific primers for the hRFC (Table 1). The results showed the PCFT/HCP1 mRNA (normalized to β-actin mRNA) to be significantly (P < 0.01) lower in cells maintained under folate-oversupplemented condition than in cells maintained under folate-sufficient condition (Figure 2D).

**Effect of folate oversupplementation on renal folate uptake**

The results on the effect of maintaining the human-derived renal epithelial HK-2 cells in folate-oversupplemented growth medium on folic acid (2 µmol/L) uptake at buffer pH 5.5 are shown in Figure 3. As can be seen, uptake by cells grown in folate-oversupplemented growth medium (100 µmol/L) was significantly (P < 0.01) lower than uptake by cells maintained in folate-sufficient growth medium (0.25 µmol/L); uptake by cells maintained in the presence of 9 µmol/L folate fall in between. However, uptake of the unrelated biotin (7.9 nmol/L) was similar under the different folate conditions (21.3 ± 1.9, 22.4 ± 0.8, and 22.2 ± 2.9 fmol · mg protein⁻¹ · 7 min⁻¹ in the cells maintained in the presence of 100, 9, and 0.25 µmol folate/L, respectively). When uptake of folic acid (2 µmol/L) was examined at buffer pH 7.4, the uptake was found to be similar under the different folate conditions (Figure 3). As reported previously for renal folate uptake (23), uptake of folic acid by HK-2 cells maintained in folate-sufficient growth medium was significantly (P < 0.01) higher at buffer pH 5.5 than at buffer pH 7.4 (0.53 ± 0.06 and 0.12 ± 0.01 pmol · mg protein⁻¹ · 7 min⁻¹, respectively).

**Effect of maintaining HK-2 cells in folate-oversupplemented growth medium on hRFC steady state mRNA and protein and on hRFC promoter activity**

The normal kidney expresses FR, RFC, and PCFT/HCP1 systems (19–24). The FR functions in the nanomolar range (20–24), whereas the hRFC and the PCFT/HCP1 systems function in the micromolar range (16, 18, 19). In this study, we determined the effect of maintaining the HK-2 cells in folate-oversupplemented growth medium on the mRNA expressions of hRFC, the PCFT/ HCP1 systems, and FR. Semiquantitative RT-PCR was performed on RNA isolated from cells grown under folate-oversupplemented and -sufficient conditions using specific primers for hRFC, PCFT/HCP1, and FR (Table 1). The results showed the mRNA of the hRFC, PCFT/ HCP1, and FR (normalized to β-actin gene) to be significantly (P < 0.01 for all) lower in cells maintained in folate-oversupplemented growth medium than in cells maintained in folate-sufficient media.

Effect of maintaining HK-2 cells in folate-oversupplemented growth medium on protein expressions of hRFC and FR was also examined by Western analysis with the use of specific polyclonal antibodies as described in “Methods.” The results showed a significant (P < 0.01) reduction in the levels of both proteins in cells maintained under folate-oversupplemented conditions than in cells maintained under folate-sufficient conditions (Figure 4, D and E).

We also examined the effect of maintaining HK-2 cells in folate-oversupplemented growth medium on activity of the hRFC promoter [a prominent hRFC promoter in a variety of tissues (31, 34)]. The results showed activity of the hRFC promoter to be significantly (P < 0.01) lower in cells maintained in folate-oversupplemented growth medium than in cells maintained in folate-sufficient growth medium.
FIGURE 4. A: Effect of folic acid oversupplementation on human reduced folate carrier (hRFC) mRNA level in HK-2 cells. Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis was performed with the use of mRNA from HK-2 cells maintained in growth media oversupplemented (100 μmol/L) or sufficient (0.25 μmol/L) in folate (as well as in the presence of an intermediate folic acid concentration of 9 μmol/L) and the hRFC specific primers as shown in Table 1. All experiments were run on at least 3 separate occasions. Results of a representative experiment are shown. Values are mean ± SEM. *P < 0.05; **P < 0.01. B: Effect of folic acid oversupplementation on folate receptor (FR) mRNA in HK-2 cells. Semiquantitative RT-PCR analysis was performed with the use of mRNA from HK-2 cells maintained in growth media oversupplemented (100 μmol/L) or sufficient (0.25 μmol/L) in folate (as well as in the presence of an intermediate folic acid concentration of 9 μmol/L) and the FR specific primers as shown in Table 1. All experiments were run on at least 3 separate occasions. Results of a representative experiment are shown. Values are mean ± SEM. *P < 0.05; **P < 0.01. C: Effect of folic acid oversupplementation on proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1) mRNA in HK-2 cells. Semiquantitative RT-PCR analysis was performed with the use of mRNA from HK-2 cells maintained in growth media oversupplemented (100 μmol/L) or sufficient (0.25 μmol/L) in folate (as well as in the presence of an intermediate folic acid concentration of 9 μmol/L) and the PCFT/HCP1 specific primers as shown in Table 1. All experiments were run on at least 3 separate occasions. Results of a representative experiment are shown. Values are mean ± SEM. *P < 0.01. D: Effect of folic acid oversupplementation on the level of hRFC protein in HK-2 cells. Western blot analysis was performed with the use of membranous fractions of HK-2 cells maintained in growth media oversupplemented (100 μmol/L) or sufficient (0.25 μmol/L) in folate (as well as in the presence of an intermediate folic acid concentration of 9 μmol/L) and specific anti-hRFC polyclonal antibodies. Image and data shown are representative of 3 separate sets of experiments. Values are mean ± SEM. *P < 0.01. E: Effect of folic acid oversupplementation on the level of FR protein in HK-2 cells. Western blot analysis was performed with the use of membranous fractions of HK-2 cells maintained in growth media oversupplemented (100 μmol/L) or sufficient (0.25 μmol/L) in folate (as well as in the presence of an intermediate folic acid concentration of 9 μmol/L) and specific anti-FR polyclonal antibodies. Image and data shown are representative of 3 separate sets of experiments. Values are mean ± SEM. *P < 0.01. F: Effect of folic acid oversupplementation on the activity of hRFC–promoter B (pB) promoter in HK-2 cells. Cells maintained in growth media oversupplemented (100 μmol/L) or sufficient (0.25 μmol/L) in folate (as well as in the presence of an intermediate folic acid concentration of 9 μmol/L) were cotransfected with hRFC-pB promoter: luciferase reporter plasmids and a control pGL3-basic vector as described in Subjects and Methods. Values are mean ± SEM; n = 3. Firefly luciferase activity was normalized relative to the activity of simultaneously expressed Renilla luciferase. *P < 0.05; **P < 0.01.
vitro models for investigating physiologic and molecular aspects of nutrient transport with results that are similar to those found in native human intestinal and renal epithelia (25, 26).

Our results with the intestinal Caco-2 cells showed that maintaining these cells in a folic acid–oversupplemented growth medium leads to a significant down-regulation in folic acid uptake (at pH 5.5 but not 7.4) compared with uptake by cells maintained under a folate-sufficient condition. The observed down-regulation was specific for folic acid because uptake of the unrelated water-soluble vitamin, biotin, was not affected by maintaining the cells under different folate conditions. The down-regulation in folic acid uptake in the folate-oversupplemented cells was associated with a decrease in the mRNA and protein levels of the hRFC system; it was also associated with a decrease in message level of the PCFT/HCP1 system. Both of these transport systems are believed to be involved in the intestinal folate uptake process (17, 19). The observed reductions of mRNA abundance of these transporters raise the possibility of involvement (at least in part) of transcriptional regulatory mechanism(s). This assumption was confirmed, at least for the hRFC system, by showing a significant reduction in the activity of the hRFC pB in Caco-2 cells maintained in folate-oversupplemented growth medium compared with cells maintained in folate-sufficient growth medium.

About the effect of folate oversupplementation on renal folate uptake, our findings with HK-2 cells showed that maintaining these cells in a folic acid–oversupplemented growth medium leads to a significant down-regulation of folic acid uptake at buffer pH 5.5 compared with cells maintained in folate-sufficient growth medium. No such down-regulation was observed when folic acid uptake was examined at buffer pH 7.4. Again the down-regulation in folic acid uptake by folate oversupplementation was specific for folic acid, because uptake of the unrelated biotin was not affected by the folate concentration in the growth medium. Unlike the intestine, the normal kidneys express the FR, the RFC, and the PCFT/HCP1 system (19–24), all of which are shown or believed to play a role in renal folate uptake process (19–24). Note that the human renal FR has an apparent Kₘ for folic acid in the nanomolar range \([\approx 10 \text{nM (23)}]\), whereas the apparent Kₘ of the hRFC and the PCFT/HCP1 is in the micromolar range \((16, 18, 19)\). It is, therefore, reasonable to assume that under our experimental conditions with \(2 \mu \text{mol/L} \) folic acid, uptake is mainly mediated via the hRFC and PCFT/HCP1 systems and that the observed down-regulation in folate uptake is mainly due to changes in the activity of these systems. Nevertheless, we have determined the effect of maintaining the HK-2 cells in folate-oversupplemented growth medium on the steady state mRNA levels of the hRFC system, the PCFT/HCP1 system, and the FR and found a significant reduction in all cases compared with cells maintained under folate-sufficient growth medium. The level of the hRFC and the FR proteins were also reduced in cells maintained under folate-sufficient condition compared with cells incubated under folate-sufficient condition. The reduction in mRNA abundance of these transporters raises the possibility that a transcriptional regulatory mechanism(s) may be involved. This assumption was confirmed, at least, for the hRFC system by showing a significant reduction of the hRFC pB activity in HK-2 cells maintained in folate-oversupplemented medium compared with cells maintained in folate-sufficient medium.

From the above discussion, it is clear that both the intestinal and the renal folate uptake processes are down-regulated on long-term oversupplementation with folic acid. This phenomenon may have significant clinical relevance when persons consuming high doses of folate experience serious acute illnesses that lead to abrupt cessation of food intake. In such circumstances down-regulation of renal tubular folate reabsorption in the absence of its continued intake can lead to precipitous depletion of this important essential nutrient at a time when its adequate supplies are critical to meet the heightened metabolic demands and reparative processes. Notable among such cases are persons experiencing catastrophic accidents, stroke, acute gastrointestinal disorders (eg, obstruction, infarction, severe gastroenteritis, acute abdominal events, etc), and fulminant infections, among others. In such cases parenteral administration of folate should be considered to avoid precipitous development of a serious deficiency state. Clinical investigations are needed to test whether this scenario does indeed occur in such patients who have been on folate supplements and to determine the time frame required for restoration of the normal intestinal and renal folate uptake processes.

In summary, results of these investigations show that long-term oversupplementation with folic acid leads to a specific and significant down-regulation of both intestinal and renal folate uptake processes. The observed down-regulation folate uptake was associated with significant reductions of hRFC, PCFT/HCP1, and FR expressions. Furthermore, at least for the hRFC system, the down-regulation appeared to be mediated (in part) via transcriptional regulatory mechanism(s).

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REFERENCES

Variants of the peroxisome proliferator-activated receptor γ- and β-adrenergic receptor genes are associated with measures of compensatory eating behaviors in young children¹–³

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ABSTRACT
Background: Young children can regulate energy precisely in the short term, showing the potential for an innate compensation mechanism of eating behavior. However, data suggest that precise compensation is attenuated as a function of increasing adiposity, parental feeding style, and age. Common variation in candidate obesity genes may account for some of the individual variation observed in short-term energy compensation. Polymorphisms in the peroxisome proliferator-activated receptor γ (PPARG) and β-adrenergic receptor (ADRB3) genes have been linked to increased body mass index (BMI; in kg/m²), obesity, and more recently dietary nutrients and preferences. In addition, common variation in ADRB3 interacts with PPARG to modulate adult body weight.

Objective: This study investigated whether variants in these genes were associated with measurable effects on child eating behavior.

Design: Children (n = 84) aged 4–10 y were prospectively selected for variants of the PPARG locus (Pro12Ala, C1431T). Heights and weights were measured. Energy intake from a test meal was measured 90 min after ingestion of a no-energy (NE), low-energy (LE), or high-energy (HE) preload, and the compensation index (COMPX) was calculated.

Results: BMI differed significantly by gene model, whereby Pro12Ala was associated with a lower BMI. Poor COMPX was associated with the PPARG T1431 allele (P = 0.009). There was a significant interaction between COMPX and the ADRB3 Trp64Arg variant in modulating compensation (P = 0.003), whereas the Arg64 allele was associated with good compensation (P = 0.001).

Conclusions: This is the first study to suggest that a genetic interaction involving ADRB3 and PPARG variants influences eating behavior in children.

KEY WORDS Children, eating behavior, energy compensation, PPARG gene variants, BMI, body mass index

INTRODUCTION
Childhood obesity has increased in the United Kingdom (1). In Scotland 5.0% of boys and 7.2% of girls aged 4–10 y were obese, and 16.1% of boys and 20.9% of girls were overweight in 2003 (2), which confirms an exponential increase in the past decade. Causes of obesity are complex, but generally they result from a sustained imbalance of energy intake over expenditure, influenced by psychological, environmental, physiologic, and genetic factors. Genetic variation may influence the partitioning of energy metabolism, may predispose to site-specific adiposity, and may influence the experience of hunger, satiety, and other regulatory mechanisms for controlling food intake (3, 4).

The ability of persons to respond to hunger and satiety cues and to resist external cues to eat is encapsulated by a measure of short-term energy compensation (5–10). Individual differences in children’s ability to regulate energy intake indicate that poor short-term energy regulation is associated with increased child age (6, 8), adiposity (11, 12), and restricted access to energy dense, highly palatable foods (11). These factors explain only part of the variance. Unexplained variation (between individuals) leads us to question the role of gene differences in short-term compensation (13–14).

One factor linking adiposity, food response, and appetite is the nuclear fatty acid receptor peroxisome proliferator-activated receptor γ (PPARγ) encoded by the PPARG gene (15). PPARγ is expressed in adipose tissue and is a key regulator of adiposity and energy balance. PPARγ is also a target for insulin-sensitizing drugs, known as thiazolidinediones (TZDs) (16). The TZD troglitazone appears to modulate appetite (17) and is mediated in part by PPARγ via regulation of leptin gene transcription (18). Notably, TZD activation of PPARγ results in down-regulation of the leptin gene (19). This association between PPARγ and leptin, a potent adiposity signal with a key role in modulating the central control of ingestive behavior, identifies PPARG as a candidate gene in mediating appetite.

Common variation of the PPARG gene has been linked to body mass index (BMI; in kg/m²) and obesity in whites. The most

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frequently studied PPARG variant is the praline-to-alanine substitution at codon 12 (Pro12Ala) (20), known to influence body weight regulation (21–24). Two additional variants, in linkage disequilibrium with Pro12Ala, are the C1431T variant in exon 6 of PPARG that is linked with susceptibility to cardiovascular disease (25, 26), leptin concentrations (27), and BMI (22, 28, 29) and the C–681G1 variant that resides in the PPARγ3 promoter region (30) and is implicated in bone growth (31) and increased height (22, 30). Diet-gene interactions among PPARGPro12Ala, dietary carbohydrate (32), and fat (33–35) also suggest a PPARG role in modulating weight. In addition, the evidence for C1431T in modulating leptin concentrations further implicates PPARG in the control of appetite (27, 36).

This study investigated whether common PPARG gene variants are associated with short-term energy compensation, as a potential behavioral correlate of obesity. We hypothesized that PPARG variants associated with increased weight and BMI would be associated with low (poor) compensation (22). We also assessed the influence of the β-adrenergic receptor (ADRB) gene on eating, given their close link to PPARG function. ADRB subtypes are candidate obesity genes (37, 38), and ADRB3 and PPARG variants interact to modulate adult body weight (39).

SUBJECTS AND METHODS

Subjects

Eighty-four prepubertal school children aged 4–10 y from 47 schools across northeastern Scotland participated in the study. The sample was largely white (95%). These children were initially recruited into a much larger study on the maintenance of energy balance (22) in which genomic DNA isolated from saliva was taken from 2454 children after child and parental consent. The 84 children in this study represent a subsample prospectively enriched for the Pro12Ala and C1431T variants of PPARG. Ethical approval for this work was granted by the Tayside Committee on Medical Research Ethics and the Fife Local Research Committee. The education department in each school authority and school head teachers also gave their approval.

DNA genotyping

DNA was prepared from mouthwash saliva pellets (40). Allelic discrimination by TaqMan assays was used to determine genotype. The probes and primers used to genotype the PPARG Pro12Ala, C1431T (29), and C–681G (22) variants were described previously. The reagents for the genotyping of the ADRB3 Trp64Arg variant were as follows: forward primer, AGGCAAACCTGCTGTCTCAGTCT; reverse primer, CATCAC- CAGGTCGGCTGCC; T allele probe, 5’FAM-CCATCGC- CTGGACTCCGAGACTCC-TAMRA; and C allele probe, 5’Tet-CCATCGCAGGGACTCCGAGACTCC-TAMRA.

All assays were performed with reagents supplied by Applied Biosystems (Foster City, CA) and with standard amplification conditions. Allelic discrimination was performed on an ABI7700 sequence detection system (Applied Biosystems).

Measurement of height and weight

Height and weight were measured on the morning of the first test condition. Standing height without shoes was measured to the nearest 0.1 cm with the use of a stadiometer (SECA, Bolton, United Kingdom). Body weight, with subjects wearing light clothing, was measured to the nearest 0.5 kg with a mechanical floor scale (SECA). BMI was calculated (2). Overweight and obesity were determined by the use of age- and sex-appropriate international cutoffs (41).

Procedure for measurement of compensation index

The procedure for measurement of compensation index (COMPX) was reported elsewhere (6). This method tests a person’s ability to adjust energy intake at a test meal in response to the energy content of preloads. Briefly, children consumed either a no-energy (NE), low-energy (LE), or high-energy (HE) preload midmorning on 3 occasions at school, followed by a test-meal lunch 1.5 h later. The order of preload administration was partially randomized; the NE control preload was consistently administered as the first condition to familiarize children with the procedure and to act as a control condition. Tests were separated by at least 1 wk. Parents were instructed to provide their child with their habitual breakfast on the morning of each test and to record breakfast details.

Preloads and test meals

Three preloads were developed and designed to vary in energy density with minimal differences in sensory properties (6). The NE control (0 kJ) was 250 mL water. The LE preload (187 kcal, or 782.78 kJ) consisted of 250 mL orange drink (200 mL water + 50 mL low-energy orange still soft drink) and 56 g low-energy-dense muffins. The HE preload (389 kcal, or 1628.35 kJ) consisted of 250 mL orange drink (200 mL water + 50 mL low-energy orange still soft drink) with the addition of 15 g maltodextrin (Maxijul; SHS International Ltd, Liverpool, United Kingdom) and 56 g regular-energy-dense muffins. Each child was required to ingest 100% of the preload at each test.

Ninety minutes after preload, children were offered a self-selected test-meal lunch consisting of cold finger food (6). The meal was prepared in quantities in excess of what the children would normally be expected to consume, and the children were invited to eat and drink as much as they wanted. A maximum of 30 min was allotted for the lunch. The average group size on each test occasion was 4, and the children sat together to consume their lunch.

Assessment of food intake

Energy intake at the test meal was assessed by weighing the food items before and after lunch and then using the manufacturers’ information to calculate the total amount of energy consumed. The precision of energy compensation was assessed by using the COMPX, which was calculated as the difference in energy intake from the test-meal lunch on any 2 occasions divided by the difference in the energy content of those preloads. The value was converted to a percentage [(change in energy intake at test meal/change between preload energy content) × 100] (11). A score of 100% represents precise (calorie for calorie) compensation. Values <100% reflect undercompensation; values >100% reflect overcompensation. Good COMPX was defined as >50%; poor COMPX was defined as <50%.

Data analyses

Genotype was analyzed with the use of dominant and codominant models, with multiple variants included in the models to test for interactions. Quantitative traits (weight, height, BMI) were
analyzed with the use of univariate general linear modeling. Age and sex were included as covariates in all models.

Energy intake and COMPX data were analyzed with the use of repeated measures analyses of variance, with age and sex as between-subject factors. Where significant main effects were obtained, post hoc multiple and pairwise comparisons with a Bonferroni correction factor were applied to determine the nature of the significance.

COMPX and genotype data were analyzed with the use of repeated measures general linear modeling, with genotypes as between-subject factors. Age and sex were used as covariates. Energy intake and genotype data were also analyzed in this way.

All data were analyzed by using SPSS for WINDOWS (version 12.0; SPSS, Chicago, IL). Results for mean COMPX and total energy intake are expressed as means ± SEM unless otherwise stated. Statistical significance was set at \( P < 0.05 \)

### RESULTS

**Subject characteristics**

Characteristics of the children are shown in Table 1. Forty-three boys and 41 girls with a mean age of 7.29 y participated in the study. In this sample, 16.7% of the boys and 17.1% of the girls were overweight and a further 2.4% of the boys and 14.6% of the girls were obese.

### Allele frequencies

Allele frequencies of the 3 PPARG variants are described in Table 2. The sample was prospectively enriched for PPARG Ala12 and T1431 carriers (\( n = 84 \)). The allele frequency of the Trp64Arg variant of the ADRB3 gene is also described (Table 2).

### BMI and genotype

The role of the individual variants was examined in relation to age, sex, height, weight, and BMI (Table 3). With the use of univariate general linear modeling, all 3 PPARG variants were included in the model. With the use of this 3-variant model, none of the variants were associated individually with BMI, although Pro12Ala just missed significance with BMI at \( P = 0.066 \). Pro12Ala was significantly associated with reduced weight (\( P = 0.007 \)) and reduced height (\( P = 0.003 \)), and C−681G was significantly associated with increased height (\( P \) for model 2 = 0.008; Table 3). There were no interactions among any of the 3 variants. Examination of individual variants in isolation showed that PPARG Pro12Ala associated individually and significantly with reduced BMI (\( P = 0.01 \)) and reduced weight (\( P = 0.025 \)) but not height (\( P \) for model 1; Table 3).

There were no significant associations with BMI, weight, and height for PPARG C1431T and C−681G when examined in isolation (\( P \) for model 1; Table 3).

When the ADRB3 gene variant Trp64Arg was added into the model (\( P \) for model 3; Table 3), the effect of Pro12Ala on weight remained significant (\( P = 0.023 \)). There was also an interaction between C1431T and Trp64Arg on weight (\( P = 0.007 \)) but no separate individual effect of C1431T or Trp64Arg on weight. Examination of variants with regard to height showed separate effects of Pro12Ala (\( P = 0.010 \)) and Trp64Arg (\( P = 0.053 \)) on height (\( P \) for model 3; Table 3) and an interaction effect between Pro12Ala and C−681G (\( P = 0.033 \)) and between C1431T and Trp64Arg (\( P = 0.006 \)). Examination of the Trp64Arg variants in isolation showed no significant effects on BMI, weight, or height (\( P \) for model 1; Table 3).

### Energy intake and COMPX

Analyses of energy intake from the test meal showed a main effect of preload (\( P < 0.001 \)), which indicated that the children adjusted intake at lunch in response to preload energy content. Energy intake differed significantly by preload (NE: 3005 ± 84 kJ; LE: 2644 ± 78 kJ; HE: 2414 ± 71 kJ; \( P < 0.001 \)). Total energy intake (energy from the test meal + preload) also differed significantly by preload (\( P < 0.001 \)), indicating that, despite the adjustment in food intake at lunch after different preloads, this adjustment failed to accommodate precisely the energy content of the preloads. Thus, total energy intake increased by preload (NE: 3090 ± 122 kJ; LE: 3523 ± 113 kJ; HE: 4061 ± 105 kJ; \( P < 0.001 \)). To determine the precision of energy compensation, COMPX was calculated and analyzed. COMPX scores showed low mean values for all preloads; however, this was subject to wide individual variation (Table 4). There were no main effects of preload, sex, or age on ability to compensate at the test meal. There was no correlation between COMPX and BMI, but BMI was positively correlated to energy intake for all preloads.
TABLE 3  
Association of variants of the peroxisome proliferator-activated receptor γ (PPARG) and β-adrenergic receptor (ADRB3) genes with height, weight, and BMI (n = 84)

<table>
<thead>
<tr>
<th></th>
<th>BMI Value</th>
<th>P for model 1</th>
<th>P for model 2</th>
<th>P for model 3</th>
<th>Weight Value</th>
<th>P for model 1</th>
<th>P for model 2</th>
<th>P for model 3</th>
<th>Height Value</th>
<th>P for model 1</th>
<th>P for model 2</th>
<th>P for model 3</th>
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</thead>
<tbody>
<tr>
<td>Pro12Ala</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro/Pro</td>
<td>18.09 ± 0.33</td>
<td>29.99 ± 0.76</td>
<td>1.28 ± 0.008</td>
<td></td>
<td>0.30</td>
<td>29.99 ± 0.76</td>
<td>1.28 ± 0.008</td>
<td></td>
<td>0.30</td>
<td>29.99 ± 0.76</td>
<td>1.28 ± 0.008</td>
<td></td>
</tr>
<tr>
<td>Pro/Ala</td>
<td>16.54 ± 0.38</td>
<td>26.74 ± 0.89</td>
<td>1.27 ± 0.010</td>
<td></td>
<td>0.001</td>
<td>26.74 ± 0.89</td>
<td>1.27 ± 0.010</td>
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<td>0.001</td>
<td>26.74 ± 0.89</td>
<td>1.27 ± 0.010</td>
<td></td>
</tr>
<tr>
<td>Ala/Ala</td>
<td>16.99 ± 0.61</td>
<td>0.010</td>
<td>0.006</td>
<td>0.121</td>
<td>0.025</td>
<td>0.007</td>
<td>0.023</td>
<td>1.29 ± 0.015</td>
<td>0.530</td>
<td>0.003</td>
<td>0.100</td>
<td></td>
</tr>
<tr>
<td>C1431T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>C/C</td>
<td>17.69 ± 0.39</td>
<td>29.27 ± 0.92</td>
<td>1.28 ± 0.01</td>
<td></td>
<td>0.001</td>
<td>29.27 ± 0.92</td>
<td>1.28 ± 0.01</td>
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<td>0.001</td>
<td>29.27 ± 0.92</td>
<td>1.28 ± 0.01</td>
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<tr>
<td>C/T</td>
<td>17.14 ± 0.31</td>
<td>28.03 ± 0.72</td>
<td>1.28 ± 0.007</td>
<td></td>
<td>0.002</td>
<td>28.03 ± 0.72</td>
<td>1.28 ± 0.007</td>
<td></td>
<td>0.002</td>
<td>28.03 ± 0.72</td>
<td>1.28 ± 0.007</td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>17.83 ± 1.10</td>
<td>0.503</td>
<td>0.587</td>
<td>0.351</td>
<td>0.549</td>
<td>0.681</td>
<td>0.164</td>
<td>0.126</td>
<td>0.999</td>
<td>0.891</td>
<td>0.433</td>
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<tr>
<td>C−681G</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>C/C</td>
<td>17.78 ± 0.36</td>
<td>29.02 ± 0.84</td>
<td>1.27 ± 0.009</td>
<td></td>
<td>0.004</td>
<td>29.02 ± 0.84</td>
<td>1.27 ± 0.009</td>
<td></td>
<td>0.004</td>
<td>29.02 ± 0.84</td>
<td>1.27 ± 0.009</td>
<td></td>
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<tr>
<td>C/G</td>
<td>17.06 ± 0.39</td>
<td>28.49 ± 0.92</td>
<td>1.29 ± 0.009</td>
<td></td>
<td>0.005</td>
<td>28.49 ± 0.92</td>
<td>1.29 ± 0.009</td>
<td></td>
<td>0.005</td>
<td>28.49 ± 0.92</td>
<td>1.29 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>17.06 ± 0.53</td>
<td>0.336</td>
<td>0.727</td>
<td>0.834</td>
<td>0.637</td>
<td>0.231</td>
<td>0.657</td>
<td>0.123</td>
<td>0.266</td>
<td>0.008</td>
<td>0.079</td>
<td></td>
</tr>
<tr>
<td>Trp64Arg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp/Trp</td>
<td>17.33 ± 0.26</td>
<td>28.25 ± 0.58</td>
<td>1.27 ± 0.006</td>
<td></td>
<td>0.003</td>
<td>28.25 ± 0.58</td>
<td>1.27 ± 0.006</td>
<td></td>
<td>0.003</td>
<td>28.25 ± 0.58</td>
<td>1.27 ± 0.006</td>
<td></td>
</tr>
<tr>
<td>Trp/Arg</td>
<td>17.63 ± 0.66</td>
<td>0.67</td>
<td>0.490</td>
<td>0.183</td>
<td>0.123</td>
<td>0.116</td>
<td>0.053</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Values are the estimated marginal $\bar{x}$ ± SEM adjusted for age and sex (ie, age and sex are covariates) after univariate general linear modeling.
2 Model 1 includes variants, one by one, individually.
3 Model 2 includes the 3 PPARG variants (Pro12Ala, C1431T, and C−681G).
4 Model 3 includes all 4 gene variants (Pro12Ala, C1431T, C−681G, and Trp64Arg).

(NE: $r = 0.37$, $P < 0.001$; LE: $r = 0.39$, $P < 0.001$; HE: $r = 0.32$, $P < 0.003$).

**COMPX and genotype**

COMPX was associated with a gene model; a main effect of PPARG C1431T ($P = 0.009$) and ADRB3 Trp64Arg ($P = 0.001$) was found for COMPX. We also tested this association adjusted for BMI, in addition to age- and sex-adjusted data, and the outcome was similar for PPARG C1431T ($P = 0.02$) and ADRB3 Trp64Arg ($P = 0.002$). PPARG Pro12Ala and C−681G were removed from the model because they did not show any contribution to this phenotype (data not shown). The effect of preload was not significant, and there was no significant interaction between preload and genotype for either variant. The main effect of genotype indicated poor COMPX (<50%) with the presence of a T1431 allele (C1431T polymorphism), whereas good COMPX (>50%) was associated with the presence of an Arg allele (ADRB3 polymorphism) (Figures 1 and 2). There was a significant interaction between Trp64Arg and C1431T in modulating COMPX ($P = 0.003$) whereby the presence of a T1431 allele ameliorated the effect of the Arg allele on COMPX (Table 5). With the use of univariate general linear modeling, a regression model was performed on the interaction between C1431T and ADRB3 gene variants for COMPX. In the resulting model for NE/HE COMPX, the overall contribution to the variance of the interaction model was 13.5%.

**Energy intake and genotype**

When the 4 genotypes (Pro12Ala, C1431T, C−681G, and Trp64Arg) were entered in the repeated measures model, energy intake from the test meal was associated with the gene model, with a main effect of C1431T ($P = 0.016$). However, this effect

![FIGURE 1. Mean (±SEM) energy compensation index (COMPX) after each preload [no-energy (NE), low-energy (LE), or high-energy (HE)] and a test meal in children carrying 1 copy (C/T) or 2 copies (T/T) of the T allele of the C1431T variant of the peroxisome-proliferator activated receptor γ gene and noncarriers (C/C). There was no genotype-by-condition interaction. Pairwise comparisons with Bonferroni adjustments between groups were as follows: P = 0.30 for NE/LE, P = 0.008 for NE/HE, and P = 0.05 for LE/HE.](image-url)
was lost when nonsignificant genotypes (Pro12Ala, C−681G, and Trp64Arg) were removed from the model.

**DISCUSSION**

The aim of the present study was to determine whether common *PPARG* gene variants influence short-term energy compensation in children, an index of eating behavior control. This work represents a novel attempt to understand the influence of genes on a specific aspect of eating behavior and appetite control.

The data on overweight and obesity show that the children in this sample were representative of the larger cohort from which these subjects were recruited (22). The data on overweight and obesity also support the presence of a genetic interaction between *ADRB3* and *PPARG* Pro12Ala variants in modulating energy compensation and a main effect of *PPARG* C1431T polymorphism, whereas good COMPX (>50%) was associated with the presence of an Arg allele. Previous studies have shown an interaction between *ADRB3* and *PPARG* Pro12Ala variants in modulating adult body weight (38), probably because of linkage with C1431T, and the Trp64Arg variant was associated with carbohydrate preferences (42). Here, we suggest the presence of a genetic interaction between *ADRB3* and *PPARG* variants in modulating energy compensation and a main effect of *PPARG* C1431T and *ADRB3*.

Interestingly, the role of *PPARG* T1431 in COMPX supports its reported association with increased BMI (29) and predisposition to cardiovascular disease (25, 26) in adults. We have also shown that this variant is associated with a small but nonsignificant increase in BMI in prepubertal children (22). In the current data, we showed no association between COMPX and child BMI; however, our children are young and will not yet express fully their *PPARG* genotype through currently developing phenotype. Thus, poor short-term energy compensation may well constitute a behavioral marker for positive energy balance later in life, as the child develops through adolescence and into adulthood. We have shown that the Pro12Ala variant is associated with lower BMI but is not associated with altered COMPX scores, which would support the notion that this Ala variant is directly associated with lower weight through modulation of energy utilization or storage, and would suggest that the mechanisms controlling eating behavior and weight gain are mediated by different genes or gene variants. In the future, we would want to test short-term COMPX effects on long-term weight gain in a longitudinal design and follow the T1431 carriers who display poor COMPX values at an early age into adulthood to determine their subsequent tendency to gain excess weight. The association of T1431 with poor satiety regulation could be explained in the context of leptin secretion and action, representing a plausible

**FIGURE 2.** Mean (±SEM) energy compensation index (COMPX) after each preload condition and test meal [no-energy (NE), low-energy (LE), or high-energy (HE)] in children carrying 1 copy (Trp/Arg) of the Arg allele of the Trp64Arg variant of the β-adrenergic receptor gene and noncarriers (Trp/Trp). There were no persons carrying 2 copies of 64Arg. There was no genotype-by-condition interaction. Pairwise comparisons with Bonferroni adjustments between groups were as follows: *P* = 0.14 for NE/LE, *P* = 0.001 for NE/HE, and *P* = 0.03 for LE/HE.
mechanism by which this variant is associated with eating behavior. Indeed the T1431 allele has been shown to be associated with altered leptin concentrations (27, 36); however, we have not measured this factor in the current study. PPARG agonists are known to play a role in regulation of leptin production; eg, TZD activation of PPARG results in down-regulation of the leptin gene (19). Low leptin concentrations in turn influence a number of neuroendocrine responses that function to conserve energy. Future analyses should include measurement of leptin concentrations to establish the strength of this link.

To date most research into the relation between specific genes and eating behavior has focused on single gene mutations and their role in appetite regulation pathways rather than on complex common gene variants. These single gene mutations, such as the leptin, leptin receptor, and melanocortin 4 receptor genes (43–47) are usually rare forms of genes that lead to severe obesity, due in part to a specific disruption or disruptions in the appetite regulatory pathway. So far, little data are available on complex common gene variants and eating behavior traits, but the potential for large interindividual variation in control of food intake that involves gene-gene and gene-environment interactions is likely. This is illustrated by recent data showing that PPARG variants interact with dietary nutrients in modulating body weight, suggesting that differential responses to dietary intake may depend on individual genotype (33–35).

The current study included a sample of persons who are largely white (95%). To check the possibility that ethnicity might have influenced the data analyses, we examined the white group (n = 80) separately in terms of allele frequency, genotype by BMI, and genotype and COMPX. The data for the white-only group were indistinguishable from the data presented here. Thus, it is unlikely that ethnic differences contributed significantly to observed gene effects. It is not yet clear how short-term markers of eating behavior predict long-term energy balance and the tendency to gain weight; hence, future follow-up studies would help elucidate our understanding of short-term energy regulation and energy balance in children.

In summary, the data presented here suggest that PPARG Pro12Ala is associated with protection from increased BMI and weight but is not associated with eating behavior specifically in terms of COMPX. PPARG T1431 may have a role in modulating COMPX in a manner concordant to its reported association with increased BMI in adults and children. It is acknowledged that common polymorphisms of the PPARG gene contribute significantly to human body weight, adiposity, and growth, and more recently data indicate that PPARG exerts modulatory effects of diet on body weight. However, this is the first study to report a relation between PPARG variants and energy compensation and supports the proposal that poor short-term energy compensation; hence, inability to regulate food intake may be a behavioral marker for future weight gain.

We thank the children, parents, and schoolteachers for their enthusiastic participation in this study and Caroline Bolton-Smith and Wendy Wrieden for their support. The author’s responsibilities were as follows—MMH, CNAP, and PW: obtained funding; JEC, BF, DJW and IM: collected the data; JEC, CNAP, and MMH: analyzed the data; JEC and MMH: wrote the paper. All authors participated in the design and conduct of the experiment and in the data interpretation and writing of this paper. None of the authors had any personal or financial conflict of interest.

REFERENCES


Effects of portion size and energy density on young children’s intake at a meal

Jennifer O Fisher, Yan Liu, Leann L Birch, and Barbara J Rolls

ABSTRACT

Background: Large portions of energy-dense foods are one feature of obesity-promoting dietary environments. Entrée portion size has been shown to influence energy intake at meals by young children. The role of energy density (ED) in children’s response to portion size, however, is unknown.

Objective: We aimed to test the effects of portion size and ED on children’s food and energy intakes at a meal.

Design: Participants were 53 (28 girls and 25 boys; 15 Hispanic, 20 black, 16 white, 2 other race) 5- to 6-y-old children [mean (±SD) body mass index percentile: = 61 ± 28]. A 2 × 2 within-subjects design was used to manipulate entrée portion size (250 compared with 500 g) and ED (1.3 compared with 1.8 kcal/g). Fixed portions of other familiar foods were provided. Weighed intake, food preference, and weight and height data were obtained.

Results: Effects of portion size (P < 0.0001) and ED (P < 0.0001) on entrée energy intake were independent but additive. Energy intake from other foods at the meal did not vary across conditions. Compared with the reference portion size and ED condition, children consumed 76% more energy from the entrée and 34% more energy at the meal when served the larger, more energy-dense entrée. Effects did not vary by sex, age, entrée preference, or body mass index z score.

Conclusions: These findings provide new evidence that portion size and ED act additively to promote energy intake at meals among preschool-aged children.

KEY WORDS

Portion size, energy density, eating behavior, children, satiation

INTRODUCTION

Exposure to large portions of energy-dense foods may contribute to childhood obesity by promoting excessive energy intake (1, 2). Secular trends reveal increases in the average size of food portions consumed by children both in and outside the home from the late 1970s through the 1990s (3). Experimental research has shown that doubling the portion size of an entrée increases preschool-aged children’s total energy consumed at the meal by 15–39% (4, 5). Recent work showed portion size effects on intake in children as young as toddlers (6). Across studies, children did not compensate for increased energy intake from large food portions by reducing the consumption of other foods. These findings suggest that large food portions can promote increased energy intake. However, evidence regarding the effects of energy density (ED; energy/g) on children’s intake is less clear.

Recent findings on the effects of ED on satiation (ie, meal termination) among young children are consistent with studies in adults (7–10). When the ED of an entrée was reduced by 30%, 2- to 5-y-olds consumed 25% fewer calories (11). Alternatively, other studies of young children have shown that satiety (ie, the intermeal interval) is heightened by increasing the ED of a preload drink consumed within 1 h of an ad libitum meal (12–14). The critical question of whether children’s response to large food portions is influenced by food ED has not been systematically addressed. Among adults, food ED and portion size have separate but additive effects on total energy consumed at meals (15) as well as over a 2-d period (16).

The main aim of this research was to test the effects of entrée portion size and ED on satiation in preschool children. We hypothesized that larger entrée portions would increase entrée and energy intake at the meal compared with smaller entrée portions. On the basis of recent findings on food ED and satiation in young children (11), we hypothesized that increasing the ED of an entrée would not affect children’s gram intake of the entrée, consequently increasing entrée energy intake. On the basis of work in adults, we hypothesized that the intake-promoting effects of portion size and ED on entrée and meal energy intake would be independent of one another (15, 16).

SUBJECTS AND METHODS

Subjects

The participants were 53 ethnically diverse (16 non-Hispanic white, 20 non-Hispanic black, 15 Hispanic, and 2 other race) 5- to 6-y-old children (28 girls, 25 boys) and their mothers living in the greater metropolitan area of Houston, TX. Exclusion criteria for the children were the presence of chronic medical conditions or medication use affecting food intake, food allergies, body mass index (BMI)-for-age <5th percentile, and dislike of the
Entrée. The convenience sample was recruited through flyers posted in the community as well through the US Department of Agriculture Children’s Nutrition Center’s website, publications, and volunteer database. The demographic characteristics of the children and their mothers are given in Table 1. On average, mothers were in their mid 30s, were overweight, and were well-educated. Children were, on average, of normal weight; 6 of 53 were overweight (BMI ≥ 95th percentile).

### Design

A 2 × 2 within-subject factorial design was used to evaluate effects of portion size and ED on satiation. Each child was seen in 4 conditions differing only in the portion size (250 or 500 g) and ED (1.3 or 1.8 kcal/g) of a macaroni and cheese entrée served at a dinner meal. Consistent with the design of previous studies in children, the reference entrée portion size was doubled for the large portion condition (4–6, 17). A design previously used to study ED among adults (9) was used as the basis of the ED manipulation in the present experiment; the ED of the dinner entrée varied by 40% across conditions via manipulations in fat and water content. Fixed portions of other foods and beverages were offered at each meal. Each condition was spaced 1 wk apart. Children ate in small groups of 3 to 4, with all children in a given group participating in the same condition sequence. The order of condition presentation was randomly assigned to each small group of children. Weighed intake methods were used to assess children’s food intake. Children’s preferences for the entrée and for other foods offered were measured by using a tasting procedure. Height and weight measurements were obtained from children and their mothers. Mothers also provided family demographic information.

### Experimental menu

A commercially available macaroni and cheese dinner (Stouffer’s, Nestle USA Inc, Solon, OH) with an ED of 1.42 kcal/g was used as the base to which butter (Land O’ Lakes Inc, Arden Hills, MN) was added to achieve an ED of 1.8 kcal/g (Table 2). Water was added to the unmodified macaroni and cheese base to create the reference ED version (1.3 kcal/g) of equal weight to the high-ED version. Quantitative affective sensory tests with untrained adults (n = 16 men, 25 women; age = 41 ± 11 y) showed no differences between versions on 100-mm visual analogue scale ratings of cheese flavor (t = 1.12, P = 0.27), color (t = 0.58, P = 0.57), oiliness (t = −0.27, P = 0.79), and pleasantness (t = −0.42, P = 0.68). Furthermore, 88% of those adults did not identify the high-ED version as containing more calories.

### Table 1

<table>
<thead>
<tr>
<th>Family demographics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Married [n (%)]</td>
<td>42 (80)</td>
</tr>
<tr>
<td>Education &gt; high school [n (%)]</td>
<td>41 (79)</td>
</tr>
<tr>
<td>Maternal age (y)</td>
<td>35.9 ± 6.2 (23–47)</td>
</tr>
<tr>
<td>Maternal BMI (kg/m²)</td>
<td>30.8 ± 6.4 (19.1–46.1)</td>
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<tr>
<td>Child BMI percentile</td>
<td>61.4 ± 28.4 (1.91–99.9)</td>
</tr>
<tr>
<td>Child BMI z score</td>
<td>0.45 ± 1.08 (−2.07–3.32)</td>
</tr>
</tbody>
</table>

1 n = 53.
2 ± SD; range in parentheses (all such values).

### Table 2

<table>
<thead>
<tr>
<th>Entrée ingredient and nutrient profiles</th>
<th>Reference energy density (1.32 kcal/g)</th>
<th>High energy density (1.84 kcal/g)</th>
</tr>
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<tbody>
<tr>
<td>Ingredients (g)</td>
<td>250-g portion</td>
<td>500-g portion</td>
</tr>
<tr>
<td>Macaroni and cheese</td>
<td>231.8</td>
<td>463.6</td>
</tr>
<tr>
<td>Water</td>
<td>18.2</td>
<td>36.4</td>
</tr>
<tr>
<td>Butter</td>
<td>—</td>
<td>18.2</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>329</td>
<td>658</td>
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<tr>
<td>Fat (g)</td>
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<td>30.8</td>
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<tr>
<td>Carbohydrate (g)</td>
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<tr>
<td>Protein (g)</td>
<td>15.4</td>
<td>30.8</td>
</tr>
<tr>
<td>Entrée ingredient and nutrient profiles</td>
<td>250-g portion</td>
<td>500-g portion</td>
</tr>
</tbody>
</table>

Entrée portion sizes were 250 and 500 g in the reference and large portion conditions, respectively. The size of the reference entrées was intended to be ample enough to avoid experimentally induced restriction of intake in the reference condition. The reference portion was similar to those used in previous studies (4, 5) and fell between the 50th and 75th percentiles for macaroni and cheese consumption by 2- to 5-yr-old children participating in the Continuing Survey of Food Intakes of Individuals (CSFII), 1994–1996 (18). The reference ED entrée provided 329 kcal in the reference portion size (250 g) and 658 kcal in the large portion size (500 g) conditions. The high-ED entrée provided 460 kcal in the reference portion size and 920 kcal in the large portion size conditions.

The portion sizes of other foods served at the dinner meal were generous and did not vary across conditions. The portion sizes were as follows: applesauce (112 g), 2% milk (1 carton; 240 g), carrots (39 g), corn (84 g), and cookies (3; 32 g). These foods, which provided 408 kcal, were included to minimize the possibility that any changes in entrée intake were attributable to limited food choice. Total energy provided in each condition was as follows: 737 kcal in the reference ED, reference portion size condition; 1066 kcal in the reference ED, large portion size condition; 868 kcal in the high-ED, reference portion size condition; and 1328 kcal in the high-ED, large portion size condition.

### Measures

#### Intake of the entrée and other foods

Children’s consumption of the entrée and other foods at the meal was measured by using weighed food intake data. Manufacturer’s data were used to convert intake in grams to energy (kcal).

#### Maternal and child weight status

Trained nurses measured height to the nearest 0.1 cm and weight to the nearest 0.1 kg, both in duplicate. The 2000 Centers for Disease Control and Prevention growth charts were used to calculate age- and sex-specific body mass index (BMI) z scores for each child (19). Child overweight was defined as a BMI z score >95th percentile. Maternal BMI was calculated as weight (kg)/height (m²).
Child food preference

The children’s preferences for the unmodified macaroni and cheese entrée as well as the other foods offered were assessed by using a tasting assessment method (20, 21). Briefly, the children were presented with 2 bites of each food, were instructed to take a taste, and were then asked to indicate their preference as being either “yummy,” “yucky,” or “just okay.” To assess familiarity, the children were asked if they had ever eaten each food.

Procedures

Height and weight were measured at the Baylor College of Medicine General Clinical Research Center in Houston, TX. The children’s intake at each dinner meal was measured at the US Department of Agriculture Children’s Nutrition Research Center’s Children’s Eating Laboratory. Parents were instructed to refrain from giving their child any foods or beverages 2 h before the visit. On arrival, a research member interviewed the parent to confirm that those instructions had been followed. An introductory visit was used to familiarize the children to the eating laboratory and to obtain food preference and anthropometric data. At all visits, 3 to 4 children were served dinner together in the presence of a research staff member. The group of children to which each child was assigned and the staff member to whom each group was assigned did not vary across visits. To minimize visual comparisons of portion sizes, each child was assigned to eat with children in the same portion size condition. Children were instructed not to share food and to eat as little or as much as desired during the 20-min timed dinner. Mothers completed self-report questionnaires in an area separate from where their children ate. All procedures were approved by the Institutional Review Board at Baylor College of Medicine and were followed in accordance with its ethical standards.

Statistical analyses

Statistical analyses were performed by using SAS version 9.1 (SAS Institute Inc, Cary, NC). Two-way factorial analyses of variance (ANOVA) were used to test effects of portion size (reference versus large), ED (reference versus high), and their interaction on children’s intake of the entrée (g, kcal), other foods (g, kcal), and meal energy (kcal). Pairwise comparisons of means across conditions were assessed by using Tukey-Kramer tests. All models were adjusted for child age, sex, ethnicity (white, Black, Hispanic, and other), child BMI z score, entrée food preference (yummy or OK), and consumption of ≥95% of the reference entrée portions. Interactions of these child characteristics with portion size and ED were also evaluated to determine whether main effects varied by those child characteristics. The significance level was set at $P < 0.05$ for main effects and $P < 0.01$ for 2-factor interactions. Data are presented as means ± SEMs unless otherwise indicated.

RESULTS

Familiarity and liking of the foods

All children reported previously eating the entrée and other foods served at the meal. Eighty-seven percent of the children ($n = 46$) rated the commercially available, unmodified version of the macaroni and cheese as tasting “yummy” and 13% ($n = 7$) rated it as tasting “just okay.” Most of the 53 children rated the other foods as tasting “yummy”: carrots ($n = 28$), applesauce ($n = 39$), cookies ($n = 44$), corn ($n = 44$), and 2%-fat milk ($n = 42$).

Consumption of the reference portions

Consumption of ≥95% of the entrée reference portions was assessed to evaluate the number of children for whom the reference serving size may have been limiting. Fourteen of the 53 children consumed ≥95% of the entrée in the reference conditions. Children who ate ≥95% of the reference portions did not consume more calories from other foods served at the meal than did the rest of the children ($P = 0.81$), but had higher total energy intakes ($P < 0.0001$). Children who ate the reference portions in full did not differ from the rest of the sample on the basis of age ($P = 0.53$), sex ($P = 0.76$), ethnicity ($P = 0.69$), or entrée preference ($P = 0.66$), but had higher BMI $z$ scores ($\bar{z} \pm SD$: $1.21 \pm 1.20$ compared with $0.18 \pm 0.90$; $P < 0.01$).

Effects of portion size and ED on children’s food and energy intakes

A main effect of portion size on entrée gram consumption was observed. Children consumed 33% more of the entrée in the large portion conditions than in the reference conditions ($210 \pm 11$ compared with $158 \pm 11$ g; $P < 0.0001$; Figure 1). Effects of portion size on gram intake of the entrée did not interact with child age ($P = 0.32$), sex ($P = 0.19$), ethnicity ($P = 0.95$), child BMI $z$ scores ($P = 0.61$), or entrée preference ($P = 0.58$). Portion size effects on entrée gram consumption were greater among children who consumed ≥95% of the reference portion entrées ($P = 0.01$), but remained significant when those children were removed from the analysis ($P < 0.05$). In contrast with portion size, entrée ED had no effect on children’s gram intake of the entrée ($P = 0.97$). Furthermore, entrée ED did not interact with portion size to influence gram intake of the entrée ($P = 0.78$). Children’s gram intake of other foods served at the meal did not vary with entrée portion size ($P = 0.57$) or ED ($P = 0.91$).

The effects of entrée portion size and entrée ED on the amount of energy that the children consumed from the entrées are depicted in Figure 2. Children consumed 33% more energy ($332 \pm 19$ kcal compared with $249 \pm 19$ kcal; $P < 0.0001$) from the entrées when served either the larger or the more energy-dense entrées than when served the reference versions. Portion size and ED did not interact to influence entrée energy intake ($P = 0.32$). Effects of portion size on children’s entrée energy intake did not interact with child age ($P = 0.46$), sex ($P = 0.19$), ethnicity ($P = 0.93$), child BMI $z$ scores ($P = 0.57$), or entrée preference ($P = 0.65$). Similarly, effects of ED on children’s entrée energy intake did not interact with child age ($P = 0.38$), sex ($P = 0.32$), ethnicity ($P = 0.88$), child BMI $z$ scores ($P = 0.38$), or entrée preference ($P = 0.64$). Both portion size ($P < 0.001$) and ED ($P < 0.01$) effects on entrée energy consumption were greater among children who consumed ≥95% of the reference portion entrées, but remained significant when those children were removed from the analysis ($P < 0.05$ and $P < 0.05$ for portion size and ED, respectively).

Compared with the reference condition, total energy intake consumed at the meal was $\approx 15\%$ higher when the large portion entrées were served ($548 \pm 19$ compared with $478 \pm 19$ kcal in
FIGURE 1. Effects of portion size and energy density (ED) on food intake. Ref, reference; Lg, large. ANOVA was used to test the effects of portion size and ED on food intake, adjusted for child’s age, sex, ethnicity, BMI z score, entrée preference, and eating ≥ 95% of the reference portions (n = 53). Data are presented as LS means (±SEMs); different letters signify mean differences, P < 0.01. Main effects of portion size on entrée intake in grams (F = 21.25, P < 0.0001) and total grams consumed at the meal (F = 9.04, P < 0.01) were observed. Portion size did not affect gram intake of other foods (F = 0.32, P = 0.57). ED did not affect gram intake of the entrée (F = 0.00, P = 0.97), other foods (F = 0.01, P = 0.91), or the total grams consumed at the meal (F = 0.00, P = 0.95). Portion size effects on food intake did not interact with ED.

FIGURE 2. Effects of portion size and energy density (ED) on energy intake. Ref, reference; Lg, large. ANOVA was used to test the effects of portion size and ED on energy intake, adjusted for child’s age, sex, ethnicity, BMI z score, entrée preference, and eating ≥ 95% of the reference portions (n = 53). Data are presented as LS means (±SEMs); different letters signify mean differences, P < 0.01. Main effects of portion size on entrée energy intake (F = 19.87, P < 0.0001) and total meal energy (F = 13.34, P < 0.001) were observed. Main effects of ED on entrée energy intake (F = 19.79, P < 0.0001) and total meal energy (F = 19.07, P < 0.0001) were observed. Neither portion size (F = 1.55, P = 0.21) nor ED (F = 0.01, P = 0.92) affected the energy intake of other foods. Portion size effects on energy intake did not interact with ED.

the large and reference portions, respectively, P < 0.001). Similarly, total meal energy was 18% higher when the more energy-dense entrées were served compared with when the less energy-dense entrées were served (554 ± 19 kcal versus 471 ± 19 kcal in large and reference portions respectively, P < 0.0001). The effect of entrée portion size on total meal energy was independent of entrée ED (F = 0.80, P = 0.37). Effects of portion size and ED on total meal energy did not interact with child age, sex, entrée preference, ethnicity, or child BMI z scores (data not shown). ED effects on meal energy were greater among children who consumed ≥ 95% of the reference portion entrées (F = 6.90, P < 0.05), but remained significant when those children were removed (F = 4.30, P < 0.05).

Compared with the condition in which the entrée was of a reference portion and ED condition, entrée energy consumption was 76% greater (382 ± 23 kcal versus 217 ± 23 kcal) and total energy intake at the meal was 34% greater (598 ± 24 kcal versus 445 ± 24 kcal) when the larger portion of the more energy dense entrée was served.

DISCUSSION

The present findings provide new evidence of elevated meal energy consumption by young children when served large portions of energy-dense entrées. In this study, the children’s intake of a dinner entrée increased by 33% when its portion size was doubled. Similarly, energy consumed from the entrée was increased by 33% when its ED was increased by 40%. The effects of portion size and ED on children’s eating were independent of one another but acted additively to promote entrée and meal...
energy consumption. Entrée energy intake was increased by \approx 75\% and total meal energy increased by almost 35\% when the entrée ED and portion size were simultaneously increased. A similarly designed adult study produced comparable findings; energy intake at the meal was 56\% greater when a large portion (900 g) of an energy-dense entrée (7.32 kJ/g) was served than when a smaller portion (500 g) of a less energy-dense entrée (5.23 kJ/g) was served (15).

The 15\% increase in total meal energy observed in the large portion conditions is consistent with previous experimental studies of children in which meal energy intake increased by 13–39\% when the portion size of a main lunch or dinner entrée was doubled (4–6). That the effects were observed even after excluding “plate cleaners” from the analyses indicates that the results were not attributable to an artificial restriction of the entrée portion size in the reference portion size conditions. Consequently, intake of the large entrée portions appeared to be excessive relative to that of the smaller portions. Limited evidence indicates that the promotion of intake by large portions may extend beyond meals to influence total daily energy intake among children. CSFII data (1994–1996, 1998) showed that the average portion size of foods consumed explained between 17\% and 19\% of the variance in daily energy intake among preschool-aged children (22). Experimental research has shown a 9\% increase in daily energy intake among African American and Hispanic preschoolers when the portion sizes of entrées and a snack served over a 24-h period were doubled (17). Adult studies have also shown portion size effects on energy intake over 2-, 23- and 11- to 24 periods, when all food and beverage portions were increased. The present results clarify implications for energy balance in children by demonstrating that large portions exerted the greatest effects on meal energy when energy-dense.

Observational studies of free-living dietary intake among young children show a positive correlation between dietary ED and daily energy intake (25, 26). The present findings are among the first to experimentally demonstrate that processes leading to meal termination (ie, satiation) by young children are relatively insensitive to food ED. Children neither consumed fewer grams of the entrée when its ED was increased by 40\% nor ate less of other foods served at the dinner meal. As a result, entrée and meal energy intakes were greater when the more energy-dense entrées were served. Adult ratings of the entrées, obtained as part of this research, indicate that the results were not likely explained by sensory differences between the reference and high-ED versions. The observed effect of entrée ED on meal energy is congruous with the findings of a recent study of 2- to 5-y-olds using a similar ED manipulation (11). In that study, children consumed 25\% less energy at meals when served a 1.4-kcal/g entrée than when served a 2.0-kcal/g entrée. The present findings are also in general agreement with adult studies; a consistent weight (volume) of food was consumed at single and multiple meals when the ED of a given menu was systematically varied (7–10; see reference 27 for a review).

The mechanisms by which ED exerts an influence on children’s energy intake remain poorly understood. In contrast with effects on satiation, experimental studies of satiety (ie, the inter-meal interval) in young children have shown that subsequent ad libitum intake is suppressed in response to increases in the ED of fixed portions of foods consumed as preloads. In that work, preschool-aged children accurately adjusted their ad libitum energy intake at a meal (12, 13) and across successive meals (28) to achieve constant energy intake across conditions of varying preload ED; this sensitivity, however, appears to decrease with age (29). After a preload, there is a greater opportunity for feedback from post-ingestive signals than in studies of meal termination during ad libitum consumption. It is possible that post-ingestive signals from food ED inhibit appetitive drive but are less tightly controlled than those processes that defend against energy deficit (30). Additional research is needed to understand the basis of apparent differences in effects of food ED on young children’s satiation and satiety.

Limited evidence suggests that overweight individuals consume larger food portions and have more energy-dense diets. Data from the CSFII, 1994–1996 and 1998, show that heavier toddlers (31) and adolescents (32), but not preschoolers (32), consumed larger food portions. A relation between child weight status and dietary ED has not been established. Among adults, however, weight status has been positively associated with dietary ED, both with (33) and without the inclusion of energy-containing beverages (34). In the present study, interactions of child weight status with portion size and ED were not significant. Consistent with previous laboratory studies (5, 6, 17), these findings indicate that the effects of portion size and ED were not moderated by child weight status. In other words, overweight and nonoverweight children appear to respond similarly to large food portions in the laboratory. As such, the observation that heavier children consume large food portions (31, 32) may reflect greater routine exposure to large portions foods rather than a weight-based susceptibility to overconsume them.

The use of a small convenience sample in this research limits the generalizability of the findings. Because preference data were obtained only for the unmodified version of the macaroni and cheese entrée, the extent to which differences in children’s intake of the reference and high-ED entrées were due to preference is not possible to discern. Adult sensory ratings of the entrées, however, suggest that any such differences were minimal. A final consideration is that 25\% of the sample consumed the reference entrée portions in full. In contrast with previous studies (5, 6), the “plate cleaners” consumed significantly greater amounts of the entrée in response to increasing portion size and ED than the rest of the children. It is not possible to discern the extent to which the intake of “plate cleaners” was restricted by the reference portions. The main findings, however, were unchanged when the data from children who consumed ≥95\% of the reference portions were excluded. Future work is needed to understand how “plate cleaners” respond to increasing food portion size.

In conclusion, this study revealed independent and additive effects of entrée portion size and ED on young children’s entrée intake and total energy consumed at the meal. These results support the perspective that large portions of energy-dense foods foster obesigenic eating behavior among young children by promoting energy intake at meals.

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Association between dietary glycemic index and age-related macular degeneration in nondiabetic participants in the Age-Related Eye Disease Study\textsuperscript{1–4}

Chung-Jung Chiu, Roy C Milton, Gary Gensler, and Allen Taylor

ABSTRACT

Background: Age-related macular degeneration (AMD) is the major cause of irreversible blindness. AMD appears to share several carbohydrate-related mechanisms and risk factors with diabetes-related diseases, including retinopathy and cardiovascular disease (CVD); however, to date, only one small study has addressed this issue.

Objective: The objective was to test the hypothesis that dietary glycemic index (dGI), which has been related to the risk of diabetes and CVD, is associated with the risk and severity of AMD in non-diabetic elderly populations.

Design: Dietary information was obtained from 4099 participants aged 55–80 y (56% women) in the Age-Related Eye Disease Study (AREDS). A total of 8125 eligible eyes at baseline were classified into 1 of 5 AMD groups according to the size and extent of drusen, the presence of geographic atrophy, and neovascular changes. We used a generalized estimating approach to evaluate the relations between dGI and risk and severity of AMD with eyes as the unit of analysis.

Results: Compared with eyes in the first quintile of dGI, eyes in the fourth and fifth quintiles had a significantly or suggestively higher risk of large drusen, geographic atrophy, and neovascularization. The multivariate-adjusted odds ratios (95% CIs) for the highest quintile were 1.42 (1.09, 1.84), 1.78 (0.81, 3.90), and 1.41 (0.95, 2.08), respectively, of which only the odds ratio for large drusen was significant. A significant positive relation between dGI and severity of AMD was also noted ($P$ for trend < 0.001). There was a 49% increase in the risk of advanced AMD (geographic atrophy plus neovascularization) for persons with a dGI higher than the sex median (women: $\geq 77.9$; men: $\geq 79.3$). This result indicated that 20% of prevalent cases of AMD would have been eliminated if the AREDS participants consumed diets with a dGI below the median.

Conclusion: The association between dGI and AMD from the AREDS cross-sectional analysis at baseline suggests that a reduction in the dGI, a modifiable risk factor, may provide a means of diminishing the risk of AMD.

KEY WORDS Retina, nutrition, carbohydrate, diabetes, insulin, cardiovascular diseases, glycation, fat, inflammation, aging, stress, epidemiology, risk factor, insulin-like growth factor

INTRODUCTION

Age-related macular degeneration (AMD) is a multifactorial, neurodegenerative disease of the central retina or macula. The macula comprises only $\approx 4\%$ of the total human retina area, but it is responsible for all our high-acuity vision. AMD usually occurs after middle age and is the leading cause of irreversible vision loss in Australian, Western European, and North American populations (1). As populations in these developed countries continue to age, this condition is emerging as a major public health issue. In the United States alone, the number of people with visually impairing AMD is expected to double and reach 3 millions by 2020 (2). There is no effective therapy for AMD, but dietary intervention (eg, antioxidant supplementation) appears to offer a means to delay the progression of this debility (3, 4). In contrast with the many studies of antioxidant nutrient intakes that have been conducted (5, 6), only one epidemiologic study of the associations between dietary carbohydrate and risk of AMD has been conducted (7). This information is particularly important because there has been an increasing trend of carbohydrate intake in the United States during the past 30 y, and one-half of our calories come from carbohydrates (8). Furthermore, increasing evidence indicates that carbohydrate can damage ocular tissues (9) and that dietary carbohydrate is associated with the risk of age-related eye diseases in nondiabetic persons (7, 10, 11). This is not surprising for the following reasons: 1) despite differences in pathological features between age-related and diabetes-related eye diseases, they share several carbohydrate-related mechanisms (9), including the formation of advanced glycation end...
products (AGE) and their sequelae (7, 9, 12, 13); 2) hyperglycemia-mediated damage can occur below the diabetic threshold in diabetes and cardiovascular disease (CVD), for example (14, 15), and AMD and CVD appear to share some risk factors (16); and 3) ocular tissues are totally dependent on the circulation for the glucose supply. Because carbohydrate nutrition has been linked to the development of diabetes and CVD, we speculate that dietary carbohydrate may also play a role in the development of AMD.

The glycemic index (GI) is a physiologic measure of the glycemic quality of carbohydrate-containing foods and can be used to guide consumers to choose foods. High-GI foods result in elevated blood glucose concentrations relative to low-GI foods. GI is defined as the ratio of area under curve of 2-h blood glucose curves from the same amount (50 g) of available carbohydrate from test food versus reference food (pure glucose or white bread) (17). The dietary GI (dGI) is a weighted average of the GIs of foods in the diet \( \sum ( \text{GI} \times W_i) / W \) (18). dGI has been implicated in the development of obesity, diabetes, CVD, and cancers (19, 20). Studies also suggest that a high dGI is associated with several components of the metabolic syndrome, such as low HDL cholesterol (21, 22), and with measures of chronic inflammation, such as elevated C-reactive protein concentrations (23), which have been related to CVD and to AMD (24–27).

Compared with the previous study, which addressed only the earliest manifestations of AMD (7), in the present study we studied the association of dGI with early and late stages of AMD in nondiabetic elderly participants in the Age-Related Eye Disease Study (AREDS; \( n = 4757 \) persons aged 55–80; 56% women) (28).

**SUBJECTS AND METHODS**

**AREDS population**

AREDS of the National Eye Institute of the National Institutes of Health (Bethesda, MD) is a long-term multicenter, prospective study dedicated to assess the clinical course, prognosis, risk factors, and prevention strategy of both AMD and cataract (28). The protocol was approved by a Data and Safety Monitoring Committee and by each Institutional Review Board for the 11 participating ophthalmic centers before initiation of the study. Participants were 55–80 y of age at enrollment and were required to have at least one eye with a visual acuity of 20/32 or better, and the lens and vitreous had to be sufficiently clear to permit good quality retinal photographs that would permit identification and quantification of small drusen. In addition, at least one eye of each participant was free from eye disease that could complicate assessment of AMD or lens opacity progression (eg, optic atrophy and acute uveitis), and that eye could not have had previous ocular surgery (except cataract surgery and unilateral photocoagulation for AMD). Finally, potential participants were excluded for illness or disorders that would have made long-term follow-up or compliance with the study protocol unlikely or difficult. A total of 4757 participants were enrolled from November 1992 to January 1998. Informed consent was obtained from participants before enrollment.

**Procedures**

Data on possible risk factors for AMD were obtained from a baseline general physical and ophthalmic examination, a detailed questionnaire on basic characteristics and demographic data, and a food-frequency questionnaire (FFQ) (29). The FFQ was validated in relation to a 24-h dietary recall in a subset of the AREDS volunteers (\( n = 192 \)). Correlations for energy and carbohydrate intakes between the 24-h dietary recall and the FFQ were 0.51 (\( P < 0.001 \)) and 0.56 (\( P < 0.001 \)), respectively (N Kurinjii, G Gensler, and R Milton, unpublished observation, 1998). Stereoscopic fundus photographs of the macula and slit lamp and red reflex lens photographs were taken and graded at a central ophthalmic photograph reading center, where the various lesions associated with AMD and the degree of lens opacities by type were assessed according to AREDS grading procedures adapted from the Wisconsin age-related maculopathy grading system and the Wisconsin System for Classifying Cataracts from Photographs, respectively (30, 31). The AREDS AMD Classification System demonstrated satisfactory reliability for detecting onset of advanced AMD and moderate to substantial agreement on various abnormalities across the AMD spectrum (32).

For AMD grading, eyes were classified into 1 of 5 groups (see below) according to the size and extent of drusen, the presence of geographic atrophy, and neovascular changes of AMD (32). The 5 groups, numbered serially and based on increasing severity of drusen or type of AMD, were defined as follows: group 1 (control), eyes had no drusen or nonextensive small drusen (\( n = 2750 \) eyes); group 2 (intermediate drusen), eyes had one or more intermediate drusen, extensive small drusen, or pigment abnormalities associated with AMD (\( n = 1806 \) eyes); group 3 (large drusen), eyes had one or more large drusen or extensive intermediate drusen (\( n = 2803 \) eyes); group 4 (geographic atrophy), eyes had geographic atrophy (\( n = 164 \) eyes); and group 5 (neovascular), eyes had choroidal neovascularization or retinal pigment epithelium detachment (\( n = 602 \) eyes).

**Study subjects**

The recruitment scheme of AREDS used in the present study is illustrated in Figure 1. Of the available 4757 subjects, we first excluded 658 persons, including 398 persons with diabetes, 99 persons with invalid calorie intakes (valid intakes ranged from 400 to 3000 kcal/d for the women and from 600 to 3500 kcal/d for the men), and 161 persons with missing nutritional, nonnutritional, and ophthalmologic covariates. This left 8125 eyes from 4757 persons aged 55–80; 56% women (2803 eyes); group 3 (large drusen), eyes had one or more large drusen or extensive intermediate drusen (\( n = 2803 \) eyes); group 4 (geographic atrophy), eyes had geographic atrophy (\( n = 164 \) eyes); and group 5 (neovascular), eyes had choroidal neovascularization or retinal pigment epithelium detachment (\( n = 602 \) eyes).

**Assessment of dietary carbohydrate variables**

A 90-item modified Block FFQ was administered to AREDS participants at baseline. The FFQ collected information about usual dietary intakes over the previous year and classified them into 9 possible response categories, ranging from “never or less than once per month” to “2 or more times per day.” The daily total carbohydrate intake of an individual was calculated by summing the product of the frequency, serving size, and carbohydrate content per serving from individual food items derived from the nutrition database of the Nutrition Coordinating Center at the University of Minnesota. The GI (17) values for foods in the FFQ were either derived from published values based on white bread as the reference food or were imputed from GI values of comparable foods (33). The dGI for each subject was calculated as the weighted average of the GI scores for each food item, with the
amount of carbohydrate consumed from each food item as the weight (18):

$$dGI = \sum (GI_j \times W_j)/W$$  \hspace{1cm} (1)

The fiber content was subtracted from the carbohydrate content. Carbohydrate and other nutritional variables were adjusted for total energy intake by using the residuals method (34).

Defining covariates

The following variables were considered as covariates in our analyses: age, sex, education level (college graduate and high school or less), race (white and others), body mass index (BMI computed from weight and height; in kg/m²), alcohol intake (g/d), smoking status (ever and never), sunlight exposure (h/d) (35), hypertension history, lens opacity, refractive error, and energy-adjusted dietary variables, including total fat, lutein and zeaxanthin, folic acid, niacin, riboflavin, thiamine, β-carotene, vitamin C, vitamin E, and zinc intakes.

Statistical methods

To evaluate the cross-sectional relation between baseline dGI and AMD risk, we used eyes with age-related maculopathy lesions (groups 2 through 5) as our cases and those in group 1 as our controls. We first described baseline characteristics, including age, race, sex, education, smoking status, BMI, sunlight exposure (h/d), hypertension history, lens opacity, refractive error, and their associations with prevalence of AMD groups. We estimated ORs relating these potential confounders to the 4 AREDS AMD groups by logistic regression analysis with the use of SAS PROC GENMOD (version 8.2; SAS Institute Inc, Cary, NC). The procedure uses the generalized estimating equation method to estimate the coefficients and to adjust the SEs of the model terms for the correlated data resulting from repeated measurements (both eyes) in the same individual (36). This accounts for the lack of independence between 2 eyes from the same individual. To evaluate whether a strong correlation existed between dGI and nutritional covariates, we calculated Pearson correlation coefficients.

To evaluate the association between dGI and total carbohydrate intake and AMD risk, participants were divided into quintile categories according to their dGI or total carbohydrate intake. For each variable, participants in the lowest 20% of the distribution made up the referent category. The cutoffs for total carbohydrate intake were 130.1, 147.2, 162.7, and 182.4 g/d for the women and 151.2, 170.6, 187.9, and 209.0 g/d for the men. The cutoffs for dGI were 73.6, 76.6, 79.1, and 81.7 for the women and 75.7, 78.3, 80.3, and 82.8 for the men. We estimated ORs from a multivariate model adjusted for those covariates mentioned above and mutually adjusted for dGI or total carbohydrate intake.

We used 2 methods to evaluate whether any interactions existed between dGI and potential effect modifiers. For those dGI quintile indicator variables with a significant or marginally significant main effect (fourth and fifth dGI quintiles), we used additive OR models to examine the potential effect modifications of age, sex, education level, smoking status, and BMI by including interaction terms in the multivariate models (37). We also tested the interactions by including a product term between the dichotomous dGI variable (evaluated as being higher or lower than the sex median: 77.9 for the women and 79.3 for the men) and the categorical variables of potential effect modifiers in the model. The effect of dGI was further evaluated in different strata of the effect modifiers. Because the case number for group 4 was too small, the interaction analyses were done in AREDS group 3 and group 5.

To evaluate whether the severity of AMD is associated with increased dGI, we first calculated the multivariate-adjusted (for those variables mentioned above and AREDS AMD group category) means for dGI according to AREDS AMD groups by using multiple linear regression with the SAS PROC MIXED procedure and REPEATED statement to adjust for correlations.
GLYCEMIC INDEX AND AMD

### Table 1
Baseline characteristics of eyes by age-related macular degeneration (AMD) group (n = 8125)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Group 1: control (n = 2750)</th>
<th>Group 2: intermediate drusen (n = 1806)</th>
<th>Group 3: large drusen (n = 2803)</th>
<th>Group 4: geographic atrophy (n = 164)</th>
<th>Group 5: neovascular (n = 602)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt; 65 y</td>
<td>839 (30.5)</td>
<td>427 (23.6)</td>
<td>504 (18.0)</td>
<td>30 (18.3)</td>
<td>91 (15.1)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>65 to &lt; 70 y</td>
<td>1185 (43.1)</td>
<td>766 (42.4)</td>
<td>1012 (36.1)</td>
<td>51 (31.1)</td>
<td>201 (33.4)</td>
<td></td>
</tr>
<tr>
<td>≥ 71 y</td>
<td>726 (26.4)</td>
<td>613 (33.9)</td>
<td>1287 (45.9)</td>
<td>83 (50.6)</td>
<td>301 (51.5)</td>
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</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Some high school or less</td>
<td>804 (29.2)</td>
<td>617 (34.2)</td>
<td>1043 (37.2)</td>
<td>77 (47.0)</td>
<td>276 (45.9)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Some college</td>
<td>817 (29.7)</td>
<td>515 (28.5)</td>
<td>877 (31.3)</td>
<td>45 (27.4)</td>
<td>176 (29.2)</td>
<td></td>
</tr>
<tr>
<td>College graduate</td>
<td>1129 (41.1)</td>
<td>674 (37.3)</td>
<td>883 (31.5)</td>
<td>42 (25.6)</td>
<td>150 (24.9)</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>2605 (94.7)</td>
<td>1718 (95.1)</td>
<td>2735 (97.6)</td>
<td>164 (100.0)</td>
<td>590 (98.0)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>145 (5.3)</td>
<td>88 (4.9)</td>
<td>68 (2.4)</td>
<td>0 (0.0)</td>
<td>12 (2.0)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>1566 (57.0)</td>
<td>1076 (59.6)</td>
<td>1621 (57.8)</td>
<td>91 (55.5)</td>
<td>0.3633</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>1372 (49.9)</td>
<td>876 (48.5)</td>
<td>1171 (41.8)</td>
<td>62 (37.8)</td>
<td>211 (35.1)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Former</td>
<td>1230 (44.7)</td>
<td>810 (44.9)</td>
<td>1480 (50.2)</td>
<td>88 (53.7)</td>
<td>306 (50.8)</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>148 (5.4)</td>
<td>120 (6.6)</td>
<td>224 (8.0)</td>
<td>14 (8.5)</td>
<td>85 (14.1)</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History</td>
<td>927 (33.7)</td>
<td>668 (37.0)</td>
<td>1113 (39.7)</td>
<td>67 (40.9)</td>
<td>273 (45.4)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0029</td>
</tr>
<tr>
<td>Bottom 20%, &lt;23.6 kg/m²</td>
<td>610 (22.2)</td>
<td>380 (21.0)</td>
<td>560 (20.0)</td>
<td>32 (19.5)</td>
<td>109 (18.1)</td>
<td></td>
</tr>
<tr>
<td>Middle 60%, 23.6 to &lt;31 kg/m²</td>
<td>1648 (59.9)</td>
<td>1132 (62.7)</td>
<td>1689 (60.3)</td>
<td>99 (60.4)</td>
<td>352 (58.5)</td>
<td></td>
</tr>
<tr>
<td>Top 20%, ≥31 kg/m²</td>
<td>492 (17.9)</td>
<td>294 (16.3)</td>
<td>554 (19.8)</td>
<td>33 (20.1)</td>
<td>141 (23.4)</td>
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<tr>
<td>Sunlight exposure¹</td>
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<td></td>
<td></td>
<td>0.2164</td>
</tr>
<tr>
<td>Bottom 20%, &lt;0.22 h/d</td>
<td>571 (20.8)</td>
<td>319 (17.7)</td>
<td>559 (19.9)</td>
<td>32 (19.5)</td>
<td>114 (18.9)</td>
<td></td>
</tr>
<tr>
<td>Middle 60%, 0.22 to &lt;1.65 h/d</td>
<td>1666 (60.6)</td>
<td>1127 (62.4)</td>
<td>1671 (59.6)</td>
<td>94 (57.3)</td>
<td>368 (61.1)</td>
<td></td>
</tr>
<tr>
<td>Top 20%, ≥1.65 h/d</td>
<td>513 (18.7)</td>
<td>360 (19.9)</td>
<td>573 (20.4)</td>
<td>38 (23.2)</td>
<td>120 (19.9)</td>
<td></td>
</tr>
<tr>
<td>Lens opacity³</td>
<td>501 (18.2)</td>
<td>372 (20.6)</td>
<td>777 (27.7)</td>
<td>52 (31.7)</td>
<td>186 (30.9)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Refractive error²</td>
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<td></td>
<td></td>
<td>0.1017</td>
</tr>
<tr>
<td>Myopic</td>
<td>577 (21.0)</td>
<td>390 (21.6)</td>
<td>573 (20.4)</td>
<td>34 (20.7)</td>
<td>99 (16.5)</td>
<td></td>
</tr>
<tr>
<td>Hyperopic</td>
<td>2173 (79.0)</td>
<td>1416 (78.4)</td>
<td>2230 (79.6)</td>
<td>130 (78.3)</td>
<td>503 (83.6)</td>
<td></td>
</tr>
</tbody>
</table>

¹ See Procedures in Subjects and Methods for descriptions of each AMD group.
² Represents the overall distributional differences between the 5 AMD groups derived by chi-square tests.
³ See Defining covariates in Subjects and Methods for definitions.

between eyes from the same individual. The mean and 95% CI for each AMD group was calculated from the predicted values of the regression model. In consideration of clinical and pathological differences between geographic atrophy and neovascularization, we evaluated the trends for group 1 → group 2 → group 3 → group 4 and group 1 → group 2 → group 3 → group 5 separately. To test linear trends across AREDS group 1 → group 2 → group 3 → group 4 and group 1 → group 2 → group 3 → group 5, we created an AMD severity variable by assigning numbers 1, 2, and 3 to group 1, group 2, and group 3, respectively, and number 4 to group 4 and group 5 and assigned the median dGI value in each group to each eye within the group. We then regressed this AMD severity variable on the median dGI variable using 2 linear regression models separately (one for group 1 → group 2 → group 3 → group 4 and the other for group 1 → group 2 → group 3 → group 5) with adjustment for those confounders mentioned above.

To calculate the population attributable fraction (PAF) of advanced AMD (group 4 plus group 5) for dGI, we first calculated the OR for those with dGI ≥ versus < the sex median. The PAF is defined as follows:

$$\text{PAF} = P_x (\text{OR} - 1)/(\text{OR} - 1 + 1)$$

where $P_x$ is the proportion of exposure in the population and the OR approximates the relative risk when the incidence of advanced AMD in the low dGI group is low (38). The $P_x$ here is 50% because sex medians have been chosen to calculate the OR for the dichotomous dGI. We used $P < 0.05$ to denote statistical significance, and all tests were 2-sided.

**RESULTS**

The distribution of baseline AREDS characteristics is shown in Table 1. The multivariate-adjusted associations (ORs and 95% CIs) between baseline characteristics and prevalence of AREDS AMD groups are shown in Table 2. Compared with group 1 (n = 2750 eyes), cases in group 2 (n = 1806) were significantly older, were less educated, and had more sunlight exposure.
exposure. Cases in group 3 (n = 2803) were significantly older, less educated, more likely to be white, more likely to be a smoker, and more likely to have a hypertension history and lens opacity. Cases in group 4 (n = 164) were significantly older, less educated, and more likely to be a smoker. As noted previously (29), cases in group 5 (n = 602) were significantly older, less educated, more likely to be white, more likely to be a smoker, more likely to have a hypertension history, more likely to have a higher BMI, and more likely to have lens opacity and hyperopia.

Although all correlation coefficients (parenthesized) between dGI and carbohydrate (−0.04), fat (0.29), vitamin C (−0.23), vitamin E (0.15), zinc (−0.043), β-carotene (−0.14), lutein and zeaxanthin (−0.08), folic acid (0.05), riboflavin (−0.24), niacin (0.22), thiamine (0.10), and energy (0.06) intakes were significant (P < 0.05), they were weakly correlated.

In the analysis of the association between dGI and AMD risk for each category of AMD, we found a generally similar pattern for AREDS groups 3, 4, and 5 (Figure 2). Compared with eyes in the first quintile of dGI, eyes in the fourth and fifth quintiles had significantly or suggestively higher risk. The multivariate-adjusted ORs (95% CIs) for the fourth and fifth quintiles, respectively, were 1.31 (1.02, 1.66) and 1.42 (1.09, 1.84) for AREDS group 3, 1.58 (0.79, 3.16) and 1.78 (0.81, 3.90) for AREDS group 4, and 1.28 (0.89, 1.84) and 1.41 (0.95, 2.08) (P = 0.0851) for AREDS group 5. The P value for the test of trend was 0.001 for group 3, 0.082 for group 4, and 0.005 for group 5. We did not find a significantly increased risk in AREDS group 2 with increased dGI.

In the analyses of interactions with sex, education, and smoking in group 3 or group 5, we found no significant interaction in either additive OR models or in stratification analyses. Using stratification analysis, we found no significant interaction with age in group 3 and 5. However, in the additive OR models, we found a marginally significantly (P = 0.099) synergistic response between the oldest age category (≥71 y) and the fifth quintile of dGI in group 3, but no significant interaction was found in group 5. In the interaction analysis with BMI using the additive OR model, we found that for BMIs ≥31 (P = 0.086) and 31–23.6 (P = 0.023), there was a synergistic response with the fifth dGI quintile on the risk for group 3. However, there was no significant interaction in the stratification analysis in group 3. In the interaction analysis in group 5, although we found no significant effect modification of BMI in the additive OR model, a significant interaction with BMI in the stratification analysis was found (P = 0.04); we found that the higher the BMI stratum, the higher the OR of neovascularization for high dGI (≥sex median): 2.45 (P = 0.001), 1.48 (P = 0.012), and 1.03 (P = 0.91) for BMI ≥31, 31–23.6, and <23.6, respectively.

In the multivariate analysis for total carbohydrate intake, no significant trend was found (Figure 3). The multivariate-adjusted mean and 95% CI of dGI for each AREDS AMD group is shown in Figure 4. The data show that the higher the severity of AMD, the higher the dGI. The multivariate-adjusted means (95% CIs) of dGI were 77.99 (77.87, 78.11) for AREDS group 1, 78.11 (77.96, 78.26) for group 2, 78.36 (78.24, 78.48) for group 3, 79.40 (78.90, 79.90) for group 4, and 79.01 (78.76, 79.26) for group 5 (Figure 4). Both of the P values for trend (group 1 → group 2 → group 3 → group 4 and group 1 → group 2 → group 3 → group 5) were <0.001.

The OR of advanced AMD (group 4 plus group 5) for those with dGI ≥ versus < the sex median was 1.49 (95% CI: 1.19, 1.85). Using this estimate in Equation 2, we computed a prevalent
DISCUSSION

The present study supports and strengthens our hypothesis that diets that provide a higher dGI are positively associated with the risk of AMD (7). We estimate that 20% (PAF) of prevalent cases of advanced AMD (group 4 plus group 5) in the AREDS cohort would be eliminated if the AREDS participants consumed diets that have dGI values below the median (38).

Previous study

Only one prior study, the cross-sectional analysis of the Nutrition and Vision Project (NVP) of the Nurses’ Health Study, related dietary carbohydrate to AMD (7). Both studies were mainly composed of whites and used the same classification system of AMD (7, 32). In comparison with participants in the NVP (mean age: 61 y), the present study had older participants (mean age: 68 y) and was much larger and thus offered a far greater number of cases and a more complete spectrum of the lesions that define AMD. In addition, the AREDS cohort allowed

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>OR (95% CI)</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>dGI Quintile</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2: Intermediate Drusen</td>
<td>1806</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3: Large Drusen</td>
<td>2803</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 4: Geographic Atrophy</td>
<td>164</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 5: Neovascular</td>
<td>602</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 2.** Associations [odds ratios (ORs) and 95% CIs] between dietary glycemic index (dGI) and prevalence of age-related macular degeneration by macular degeneration group. Participants were divided into quintile categories according to their dGI; those in the lowest 20% of the distribution comprised the referent category. The cutoffs were 73.6, 76.6, 79.1, and 81.7 for the women and 75.7, 78.3, 80.3, and 82.8 for the men. The multivariate-adjusted logistic models using group 1 (n = 2750) as a control were adjusted for age, sex, race, education, smoking status, BMI, sunlight exposure, hypertension, lens opacity, refractive error, and energy-adjusted dietary fat, lutein and zeaxanthin, folic acid, niacin, riboflavin, thiamine, β-carotene, vitamin C, vitamin E, zinc, and total carbohydrate intakes.

**Total Carbohydrate Intake Quintile**

**FIGURE 3.** Associations [odds ratios (ORs) and 95% CIs] between total carbohydrate intake and prevalence of age-related macular degeneration by macular degeneration group. Participants were divided into quintile categories according to their total carbohydrate intake; those in the lowest 20% of the distribution comprised the referent category. The cutoffs were 130.1, 147.2, 162.7, and 182.4 g/d for the women and 151.2, 170.6, 187.9, and 209.0 g/d for the men. The multivariate-adjusted logistic models using group 1 (n = 2750) as a control were adjusted for age, sex, race, education, smoking status, BMI, sunlight exposure, hypertension, lens opacity, refractive error, dietary glycemic index, and energy-adjusted dietary fat, lutein and zeaxanthin, folic acid, niacin, riboflavin, thiamine, β-carotene, vitamin C, vitamin E, and zinc intakes.

PAF of 20% for advanced AMD for high dGI in the AREDS cohort at baseline.
us to further evaluate the interactions with other risk factors. Although the NVP collected previous long-term dietary data, the AREDS evaluated the dietary data for the previous year. However, the present findings are consistent with the findings in the NVP (7). Drusen and pigmentary abnormality are considered early signs of AMD and strong predictors of subsequent advanced AMD (39). In the NVP study, a significantly positive association was noted between dGI and pigmentary abnormality, but we could not associate intermediate drusen with dGI, seemingly because of a lack of power (7). In the present study, pigmentary abnormality was classified into AREDS group 2. This precluded the specific associations with pigmentary abnormality. However, we had much more statistical power to evaluate the associations of interest than was possible in the NVP study, especially for drusen and advanced AMD. In the present study, we found positive associations between dGI and large drusen (group 3) and advanced AMD (group 4 plus group 5). Given the results that indicate a significantly positive relation between dGI and severity of AMD, we speculate that dGI plays a role in both the early and late stages of AMD.

In the NVP, a one-unit difference in dGI was observed between early AMD (pigmentary abnormality) cases and controls, based on the means of the previous 10-y diet (7). Our present data indicate that advanced AMD cases have a mean dGI that is up to 1.4-units higher than that of controls, based on the diet over the previous year (Figure 4). According to Equation 1, the difference in dGI (ΔdGI) can be calculated from \( \Delta dGI = \frac{\sum (GI_i \times W_i)}{W} \). For a person consuming 3 servings of bread (14 g available carbohydrate per serving) in a diet containing 250 g total available carbohydrate per day, a 1.4-unit reduction in dGI is achievable by replacing the 3 servings of white bread (\( GI = 100 \), food item no. 101 (34)) with 3 servings of whole-grain bread (\( GI = 89 \), food item no. 123 (34)) from the daily diet, where \( \Delta dGI = \frac{(100 \times 14 \times 3) - (89 \times 14 \times 3)}{250} = 1.85 \). However, the difference in dGI between cases and controls may be an underestimate for the diet change associated with the reduced risk of AMD, likely because of survival bias. This is because dGI has been positively related to several major causes of mortality (19, 20). Prospective studies are needed to refine these data to arrive at dietary recommendations.

### Possible mechanisms

Much evidence supports our hypothesis. First, the blood retina barrier expresses high concentrations of glucose transporters to satisfy the large demand for glucose metabolism and this facilitates the formation and accumulation of AGE (9, 40). AGE are thought to have deleterious effects on the activity of degradative enzymes, on retinal pigment epithelium function, and on the integrity of the choriocapillaris and Bruch’s membrane (9). These biochemical and physiologic compromises could be especially pronounced after the consumption of high-GI foods, which induce an abrupt increase in blood glucose concentrations and thus may supply an excess amount of glucose relative to demand during the postprandial stage.

Second, all forms of drusen appear to have similar carbohydrate components and AGE accumulate in drusen with age and occur at a higher level in patients with advanced AMD (41). Third, a high-GI diet has been proposed to play a role in producing oxidative stress and exacerbating proinflammatory processes (19, 23, 42, 43). Oxidative insults may decrease the efficacy of the quality-control machinery in the retina (44). In addition, other hyperglycemia-mediated damage, including inflammatory and angiogenic responses that occur in advanced AMD, was found to at least partially explain AMD pathogenesis (45). It is also likely that the compensatory hyperlipidemia that follows hypoglycemia in the late postprandial stage after the consumption high-GI

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**FIGURE 4.** Multivariate-adjusted mean dietary glycemic index (dGI) by age-related macular degeneration (AMD) groups. Error bars represent 95% CIs. Multiple linear regression was used and adjusted for age, race, sex, education, smoking status, BMI, sunlight exposure, hypertension, lens opacity, refractive error, AMD group, and energy-adjusted dietary fat, lutein and zeaxanthin, folic acid, niacin, riboflavin, thiamine, \( \beta \)-carotene, vitamin C, vitamin E, zinc, and total carbohydrate intakes. \( P \) for trend values were <0.001 for control→intermediate drusen→large drusen→geographic atrophy and for control→intermediate drusen→large drusen→neovascular.
foods, and which is thought to be important in the development of CVD (19), may also play some role in AMD pathogenesis because hyperlipidemia has been implicated in the pathogenesis of AMD (26). Finally, the insulin-like growth factor axis, which plays an essential role in cell proliferation and differentiation and complements the metabolic effects of insulin, has been linked to aging and age-related diseases, including diabetes, cancers, CVD, retinopathy, as well as AMD (20, 46–48). Interestingly, recent evidence suggests that dGI could, through modulating the insulin-like growth factor axis, affect the risk of age-related diseases (20, 46). Further studies are needed to clarify the mechanism.

In the interaction analysis, we found a potential synergistic effect between old age and high dGI on the risk for group 3 (large drusen/intermediate drusen). This finding implies that dGI may partially, in addition to the biological compromise with age itself, explain the observation that drusen accumulates with age. A synergistic response was also noted between high BMI and high dGI in Group 3. In the stratification analysis of group 5, we found that the ORs for high dGI were larger in the high-BMI strata than in the low-BMI strata, which implies that dGI has a relatively higher effect on the risk of neovascularization in persons with a high BMI. However, because of inherent limitations in studying interaction with the use of epidemiologic data, studies adopting different designs and settings are needed to clarify the detailed mechanisms and implications (37).

Strengths and limitations

Using participants from a well-characterized cohort, we were able to use the standardized collection of risk factor information and photographic grading of maculopathy. Using eyes as the unit of analysis, we increased our power, which improved our ability to adjust for many previously identified risk factors and potential dietary confounders and to evaluate the interactions of interest in our multivariate analysis. This approach was further supported by the observation that, despite the difference in the unit of analysis [by person in the previous AREDS report (29) and by eye in the present study], the risk factor profiles were very similar in this study and in the previous AREDS report. Old age, lower education, and smoking were the 3 most important risk factors for AMD in the AREDS cohort. Recall and selection bias in the AREDS were unlikely to explain our findings because exposure information was collected before outcome evaluation, and our retinal classifications were performed in an independent center by graders masked to our nutrition data (11). Although GI values are generally reproducible from place to place, there are some variations in published GI values for apparently similar foods (33). For these foods, we chose the GI of the most popular American food item in our compilation (11). It is unlikely that the nondifferential misclassification in our GI compilation could explain our findings because our compilers were blinded to the ophthalmic data. Consistency with prior evidence reduced the possibility that the present findings were due to chance. Residual confounding is a concern but should be minimized because we included all known dietary and nondietary confounders in our analysis. The low correlations between dGI and other nutritional covariates indicated that it is unlikely that any other single nutrient or dietary pattern could totally account for the independent effect of dGI on the risk of AMD. The cross-sectional nature of this study limited its strength in defining causality and dietary recommendation.

Conclusions

In summary, these cross-sectional analyses suggest that poor dietary carbohydrate quality as defined by dGI, a modifiable risk factor, may increase the risk of AMD through several common etiologic factors of diabetes and CVD, including the formation of AGE and increases in oxidative stress, inflammation, and hyperlipidemia. Our results also suggest that the quality, but not the quantity, of dietary carbohydrate influences the risk of AMD in both the early and late stages of the disease. Prospective studies are needed before dietary carbohydrate management is recommended as another strategy for the prevention of AMD.

The authors’ responsibilities were as follows—C-JC and GG: had full access to all of the data and took responsibility for the integrity of the data and the accuracy of the data analysis; C-JC, RCM, and AT: conceived and designed the study, analyzed and interpreted the data, and critically analyzed the manuscript for important intellectual content; RCM: acquired the data; C-JC and AT: drafted the manuscript; CJC: conducted the statistical analysis; GG: provided administrative, technical, and material support; RCM and AT: supervised the study; all authors: contributed substantially to the manuscript. No conflicts of interest were declared.

REFERENCES

Plasma phospholipid and dietary fatty acids as predictors of type 2 diabetes: interpreting the role of linoleic acid

Allison M Hodge, Dallas R English, Kerin O’Dea, Andrew J Sinclair, Maria Makrides, Robert A Gibson, and Graham G Giles

ABSTRACT

Background: Dietary fatty acids may be associated with diabetes but are difficult to measure accurately.

Objective: We aimed to investigate the associations of fatty acids in plasma and diet with diabetes incidence.

Design: This was a prospective case-cohort study of 3737 adults aged 36–72 y. Fatty acid intake (kJ) and plasma phospholipid fatty acids (%) were measured at baseline, and diabetes incidence was assessed by self-report 4 y later. Logistic regression excluding (model 1) and including (model 2) body mass index and waist-hip ratio was used to calculate odds ratios (ORs) for plasma phospholipid and dietary fatty acids.

Results: In plasma phospholipid, positive associations with diabetes were seen for stearic acid [OR model 1, highest versus lowest quintile: 4.14 (95% CI: 2.65, 6.49), P for trend < 0.0001] and total saturated fatty acids [OR model 1: 3.76 (2.43, 5.81), P for trend < 0.0001], whereas an inverse association was seen for linoleic acid [OR model 1: 0.22 (0.14, 0.36), P for trend < 0.0001]. Dietary linoleic [OR model 1: 1.77 (1.19, 2.64), P for trend = 0.002], palmitic [OR model 1: 1.65 (1.12, 2.43), P for trend = 0.012], and stearic [OR model 1: 1.46 (1.00, 2.14), P for trend = 0.030] acids were positively associated with diabetes incidence before adjustment for body size. Within each quintile of linoleic acid intake, cases had lower baseline plasma phospholipid linoleic acid proportions than did controls.

Conclusions: Dietary saturated fat intake is inversely associated with diabetes risk. More research is required to determine whether linoleic acid is an appropriate dietary substitute.


KEY WORDS Diabetes, dietary fats, fatty acids, phospholipids, prospective study

INTRODUCTION

Our understanding of associations between dietary fats and type 2 diabetes is limited by the accuracy of measurement of habitual fat intake. To overcome this, 3 prospective studies measured fatty acid biomarkers, but none of those studies simultaneously measured diet (1–3). Consistent observations across the 3 studies were that the incidence of diabetes is associated with lower proportions of linoleic acid in plasma phospholipids (1, 3), in cholesterol esters (1), or as serum esterified and nonesterified linoleic acid (2). Two of the studies also linked higher proportions of saturated fatty acids (SFAs) with diabetes (1, 3).

These observations suggest that a low intake of linoleic acid may increase diabetes risk. A review of fat types and diabetes risk concluded that polyunsaturated fatty acids (PUFAs) could be beneficial (4), which is consistent with the above biomarker studies, although long-chain n–3 PUFAs may be particularly related to lower diabetes risk. Dietary SFAs are generally considered to have an adverse effect on insulin action and diabetes risk (4, 5), although the association between dietary and biomarker concentrations is not direct as the result of endogenous production (6).

Our aim was to investigate prospectively associations between both plasma phospholipid and dietary fatty acids and diabetes. We specifically tested the hypotheses that SFAs would be positively associated with diabetes and that linoleic acid would be inversely associated with diabetes. We also assessed whether associations with biomarkers were similar to those with dietary intakes.

SUBJECTS AND METHODS

Subjects

The Melbourne Collaborative Cohort Study (MCCS) recruited 41 528 persons (17 049 men) between 1990 and 1994. Persons aged 40–69 y were invited; 0.7% of the participants fell outside this age range but are included in all analyses. The Cancer Council Victoria, Melbourne, Australia (AMH, DRE, and GGG); the School of Population Health, University of Melbourne, Melbourne, Australia (AMH, DRE, and GGG); the Department of Medicine, University of Melbourne, St Vincent’s Hospital, Melbourne, Australia (KO’D); the School of Exercise and Nutrition Sciences, Deakin University, Melbourne, Australia (AJS); the Child Health Research Institute, Women’s and Children’s Hospital and Flinders Medical Centre, Adelaide, Australia (MM); the School of Paediatrics and Reproductive Health, University of Adelaide (MM and RAG); and the School of Agriculture, Food and Wine, University of Adelaide, Adelaide, Australia (RAG).

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2 Cohort recruitment was funded by VicHealth and The Cancer Council Victoria. This study was funded by grants from the National Health and Medical Research Council (209057, 126403) and was further supported by infrastructure provided by The Cancer Council Victoria.

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Accepted for publication February 6, 2007.
Case-cohort design

We used a case-cohort design (7) for biomarker studies. Plasma phospholipid fatty acids were measured for all incident cases of diabetes and a random sample of the cohort (the subcohort), which included some randomly selected cases. We excluded participants with diabetes at baseline (self-reported or elevated plasma glucose), those who had had a heart attack or had angina before baseline, those who did not report diabetes at baseline but later reported a date of diabetes diagnosis before baseline, those with extreme self-reported energy intakes (<1st percentile and >99th percentile), and those with missing values for relevant risk factors. A total of 3737 participants aged between 36 and 72 y, including 364 incident cases of type 2 diabetes, had complete data for these analyses.

Baseline measurements

Plasma glucose was measured by using a Kodak Ektachem analyzer (Rochester, NY). According to the World Health Organization criteria current at the time, elevated plasma glucose was defined as a concentration >7.8 mmol/L when fasting (68% of participants) and >11.1 mmol/L if not (8).

Dietary data were collected with a self-administered 121-item food-frequency questionnaire specifically developed for the MCCS (9). Information on other risk factors was collected in face-to-face interviews (10–12). Height, weight, and waist and hip circumferences were measured directly; and body mass index (BMI, in kg/m²) and waist-hip ratio (WHR) were calculated. Dietary fatty acid intake was calculated from the food-frequency questionnaire data by using Australian fatty acid composition data. The Australian database contains fatty acid data (g/100 g) to 2 decimal places for 1044 Australian foods that were selected as being of particular interest to the researchers who performed the assays; 11 SFAs, 7 monounsaturated fatty acids (MUFAs), 10 PUFAs, and 3 trans fatty acids are included (13).

Analysis of the fatty acid composition of plasma phospholipids

Blood was collected from all participants into sodium-heparin evacuated tubes, centrifuged immediately (3000 rpm, 15 min, 20°C), portioned into aliquots, and stored in liquid nitrogen. The fatty acid analysis has been described in detail elsewhere (6). Briefly, samples were realiquotted on ice under red light conditions before being refrozen and transported to the laboratory of one of us (RG) in Adelaide. Total lipids were extracted from plasma, and the extracts were separated by thin-layer chromatography into phospholipids, triacylglycerol, and cholesterol esters on silica gel plates (silica gel 60H; Merck, Darmstadt, Germany). Phospholipid fatty acid methyl esters were separated and quantified with a Hewlett-Packard (Palo Alto, CA) 5880 gas-liquid chromatograph by using a capillary column equipped with flame ionization detection and the Hewlett-Packard Chem-Station data system.

Plasma insulin measurement

Plasma insulin was measured in the plasma of fasted participants only (68%) by use of the AxSYM Microparticle Enzyme Immunoassay (Abbott, North Ryde, NSW, Australia). This assay has minimal cross-reactivity with proinsulin (0.016%) and no detectable cross-reactivity with C-peptide or glucagon.

Ascertaining of new cases of diabetes

Approximately 4 y after baseline, the participants completed a mailed, self-administered questionnaire that covered diagnosis of diabetes (10, 11). We attempted to verify with the person’s nominated doctor any reports of diabetes diagnosed since baseline. Responses were available for 292 persons; 291 (84%) were confirmed as having type 2 diabetes. For the 52 persons with no response and for the 2 persons for whom doctors did not know diabetes type, we assumed a diagnosis of type 2 diabetes.

Statistical analysis

Means and SDs for each fatty acid in plasma phospholipid and diet were calculated by diabetes status at follow-up, and t tests were used to evaluate differences between the 2 groups. Age, country of birth, sex, physical activity score, 5-y weight change, education level, smoking, BMI, WHR, and family history of diabetes were considered as potential confounders. Weight change, education, and smoking were not associated with diabetes in the subcohort and were not included in subsequent models. Logistic regression models were computed first with age, sex, country of birth, physical activity, family history of diabetes, and alcohol intake (model 1) and then with all confounders plus BMI and WHR (model 2) for quintiles (based on the distributions in the subcohort) of plasma phospholipid fatty acid proportions and dietary fatty acids expressed as energy density. The following fatty acids and classes were analyzed: total SFAs, 15:0, 16:0, 18:0, total MUFAs, 16:1n-7, 18:1n-9, total PUFAs, total n-6 fatty acids, 18:2n-6, 20:3n-6, 20:4n-6, total n-3 fatty acids, 18:3n-3, 20:5n-3, 22:5n-3, 22:6n-3, ratio of n-6 to n-3 fatty acids, total trans fatty acids, and total conjugated linoleic acid (plasma only). γ-Linolenic acid was not included because its measurement in plasma was not considered to be reliable owing to its extremely low concentrations in a substudy (6). Fatty acid ratios in plasma phospholipid reflecting product-precursor ratios of elongase and desaturase enzymes were also calculated and examined in the same way as the fatty acids. Additional analyses were performed with adjustment for insulin in subjects who were fasting at baseline. An interaction term for dietary linoleic acid and insulin was tested in model 1. Interactions between dietary linoleic acid and both BMI and age were also tested in view of the observations of van Dam (14), which showed an inverse association between linoleic acid and diabetes in younger, leaner persons.

Fatty acid reliability study

The reliability of plasma phospholipid and dietary fatty acid composition was assessed as described previously (6).

RESULTS

The baseline characteristics of the incident diabetes cases and controls in the subcohort are shown in Table 1. Persons who developed diabetes tended to be older, more obese, less active, and more likely to have a family history of diabetes and to originate from southern Europe.
Mean daily intakes of total fat and fatty acid classes by diabetes status at follow-up are presented in Table 3. Persons who developed diabetes had higher intakes of total fat, total monounsaturated fats, 16:1n−7, 18:1n−9, total polyunsaturated fats, n−6 fats, 18:2n−6, 20:4n−6, n−3 fats, 18:3n−3, and trans fats and a lower intake of 15:0 at baseline than did persons who did not develop diabetes.

The ORs for diabetes by quintile of plasma phospholipid fatty acids relative to the lowest quintile are shown in Table 4. After adjustment for age, sex, country of birth, physical activity, family history of diabetes, and alcohol intake (model 1), inverse associations were seen for 15:0, trans fatty acids, and 18:2n−6. Positive associations were observed for 18:0, total SFAs, 16:1n−7, and 20:3n−6. After further adjustment for body size, these associations were attenuated but still highly significant.

The ORs for elongation and desaturation product-precursor ratios are presented in Table 5. Strong positive associations were observed for stearoyl-CoA desaturase (ratio of 16:1n−7 to 16:0) and elongase (ratio of 20:3n−6 to 18:2n−6), whereas inverse associations were seen for Δ3 desaturase (ratio of 20:4n−6 to 20:3n−6) and the ratio of 18:1n−9 to 18:0, which also reflects stearoyl-CoA desaturase-1.

The ORs by quintile of dietary fatty acids are shown in Table 6. For model 1, the OR for the top quintile of dietary fat intake was elevated compared with the lowest. Both 16:0 and 18:0, but not total SFAs, were associated with higher risk in model 1. 16:1n−7 showed a weak positive association with diabetes. Positive associations were seen for 18:1n−9, MUFA, 18:2n−6, total n−6 fatty acids, PUFAs, and 18:3n−3 in model 1; after adjustment for body size, however, these were no longer significant. The ratio of n−6 to n−3 fatty acids showed a positive

### Table 1
Baseline characteristics of persons with (cases) or without (controls) incident diabetes, Melbourne Collaborative Cohort Study, 1990–1994

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 3391)</th>
<th>Cases (n = 346)</th>
<th>p&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>54.5 ± 8.6&lt;sup&gt;1&lt;/sup&gt;</td>
<td>57.9 ± 7.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>26.5 ± 4.2</td>
<td>31.7 ± 5.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.84 ± 0.09</td>
<td>0.91 ± 0.09</td>
<td>0.0001</td>
</tr>
<tr>
<td>Alcohol intake (g/d)</td>
<td>13.4 ± 20.4</td>
<td>11.8 ± 25.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>Women [n (%)]</td>
<td>1918 (56.6)</td>
<td>170 (49.1)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Country of birth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Australia [n (%)]</td>
<td>2425 (71.5)</td>
<td>154 (44.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>United Kingdom [n (%)]</td>
<td>256 (7.6)</td>
<td>29 (8.4)</td>
<td>0.982</td>
</tr>
<tr>
<td>Italy [n (%)]</td>
<td>397 (11.7)</td>
<td>90 (26.0)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Greece [n (%)]</td>
<td>313 (9.2)</td>
<td>73 (21.0)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Gained weight in past 5 y [n (%)]</td>
<td>793 (23.4)</td>
<td>118 (34.1)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Family history of diabetes [n (%)]</td>
<td>598 (17.6)</td>
<td>118 (34.1)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Current smoker [n (%)]</td>
<td>371 (10.9)</td>
<td>46 (13.3)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Primary education only [n (%)]</td>
<td>528 (15.6)</td>
<td>118 (34.1)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Most active [n (%)]</td>
<td>829 (24.4)</td>
<td>48 (13.9)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

<sup>1</sup> All values are x ± SD, n = 3737. CLA, conjugated linoleic acid.

<sup>2</sup> p With unequal variance.
association of borderline significance that was not attenuated by adjustment for body size.

We observed previously that plasma and dietary linoleic acid were correlated in the subcohort (6); thus, our observations here that plasma phospholipid linoleic acid was associated with diabetes risk in the opposite direction (negative) to the association observed for diet (positive) is unlikely, this would not introduce bias (15). The use of a single plasma phospholipid fatty acid measurement to reflect long-term status is a concern, but unless screening was associated with plasma phospholipid fatty acids, which is unlikely, this measurement error in the dietary estimates. To further explore how these opposite associations could occur, we plotted plasma phospholipid linoleic acid for quintiles of dietary intake for persons who developed diabetes and for those who did not. We also fitted a linear regression model with plasma phospholipid linoleic acid as the dependent variable with quintiles of dietary linoleic acid as the independent variables. An interaction between dietary linoleic acid and diabetes status was also included to test for different slopes in cases and controls. As indicated in Figure 1, for both persons who developed diabetes and for those who did not, there were similar linear associations between dietary and plasma phospholipid linoleic acid; the interaction term was not significant ($P = 0.6$ when quintile medians were modeled). However, within each quintile of reported dietary intake, persons who developed diabetes had lower mean plasma phospholipid linoleic acid proportions; the mean difference, estimated from the regression, was 1.8 (95% CI: 1.4, 2.1) percentage points.

Models were recomputed for the fasting subgroup ($n = 2324$, cases $= 224$). There was a weak interaction between plasma insulin and linoleic acid intake ($P$ for interaction $= 0.09$), and the association between dietary linoleic acid and diabetes risk was most apparent in persons with plasma insulin concentrations at or above the median value ($\geq 5.3$ pmol/L). The OR for quintile 5 versus quintile 1 was 1.81 (95% CI: 1.01, 3.23; $P$ for trend $= 0.02$), compared with lower values ($P$ for trend $= 0.30$). There was no significant difference in the associations of dietary linoleic acid with incident diabetes across strata of age ($P$ for interaction $= 0.292$) or BMI ($P$ for interaction $= 0.252$).

The 12-mo reliability coefficients (within-subject variation) for plasma phospholipid fatty acids ranged from 0.23 for palmitoleic acid to 0.89 for palmitic acid and from 0.32 for dietary palmitoleic acid to 0.49 for dietary oleic acid (6).

### DISCUSSION

We found positive associations between the incidence of diabetes and SFAs in plasma phospholipid and diet. Plasma phospholipid linoleic acid was inversely, and dietary linoleic acid was positively, associated with diabetes risk. Persons who developed diabetes had lower plasma phospholipid linoleic acid proportions for each quintile of linoleic acid intake than did persons without diabetes.

Our study has several strengths: it included both men and women and had a good follow-up rate. The trivial differences between those who did and did not complete follow-up mean that response bias was likely to be minimal. Plasma glucose was measured at baseline, and persons with elevated glucose were excluded. Although we did not attempt to verify negative reports of diabetes, participants were accurate in reporting diagnoses. Because we did not screen for diabetes at follow-up, some incident cases could be missed, but unless screening was associated with plasma phospholipid fatty acids, which is unlikely, this would not introduce bias (15). The use of a single plasma phospholipid fatty acid measurement to reflect long-term status is a potential limitation, but 12-mo reliability coefficients (within-subject variation) for a small group from the MCCS suggest that reliability was reasonable for the fatty acids studied (6).

### TABLE 3

Baseline intakes of dietary fat and fatty acids of persons with (cases) or without (controls) incident diabetes after 4 y of follow-up, Melbourne Collaborative Cohort Study, 1990–1994

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Controls</th>
<th>Cases</th>
<th>$P^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total fat</strong></td>
<td>82.16 ± 30.52</td>
<td>86.25 ± 34.22</td>
<td>0.033</td>
</tr>
<tr>
<td><strong>Saturated fatty acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15:0</td>
<td>31.38 ± 12.31</td>
<td>31.94 ± 13.53</td>
<td>0.457</td>
</tr>
<tr>
<td>16:0</td>
<td>0.27 ± 0.14</td>
<td>0.25 ± 0.14</td>
<td>0.041</td>
</tr>
<tr>
<td>18:0</td>
<td>16.28 ± 6.04</td>
<td>16.96 ± 6.78</td>
<td>0.071</td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:1n−7</td>
<td>7.79 ± 3.14</td>
<td>8.06 ± 3.50</td>
<td>0.160</td>
</tr>
<tr>
<td>18:1n−9</td>
<td>28.67 ± 10.76</td>
<td>31.60 ± 12.99</td>
<td>0.001</td>
</tr>
<tr>
<td>Polysaturated fatty acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total n−6</td>
<td>11.34 ± 4.99</td>
<td>12.20 ± 6.46</td>
<td>0.010</td>
</tr>
<tr>
<td>18:2n−6</td>
<td>11.28 ± 4.98</td>
<td>12.20 ± 6.46</td>
<td>0.010</td>
</tr>
<tr>
<td>20:3n−6</td>
<td>0.02 ± 0.02</td>
<td>0.03 ± 0.03</td>
<td>0.078</td>
</tr>
<tr>
<td>20:4n−6</td>
<td>0.041 ± 0.03</td>
<td>0.045 ± 0.03</td>
<td>0.018</td>
</tr>
<tr>
<td>Total n−3</td>
<td>1.25 ± 0.48</td>
<td>1.35 ± 0.53</td>
<td>0.002</td>
</tr>
<tr>
<td>18:3n−3</td>
<td>0.95 ± 0.33</td>
<td>1.03 ± 0.44</td>
<td>0.0006</td>
</tr>
<tr>
<td>20:5n−3</td>
<td>0.10 ± 0.09</td>
<td>0.10 ± 0.07</td>
<td>0.568</td>
</tr>
<tr>
<td>22:5n−3</td>
<td>0.03 ± 0.03</td>
<td>0.03 ± 0.03</td>
<td>0.587</td>
</tr>
<tr>
<td>22:6n−3</td>
<td>0.18 ± 0.15</td>
<td>0.18 ± 0.11</td>
<td>0.509</td>
</tr>
<tr>
<td>n−6:n−3</td>
<td>9.17 ± 2.81</td>
<td>9.16 ± 2.83</td>
<td>0.935</td>
</tr>
<tr>
<td>Total trans</td>
<td>0.10 ± 0.11</td>
<td>0.12 ± 0.13</td>
<td>0.004</td>
</tr>
</tbody>
</table>

1 All values are $\bar{x}$ ± SD; $n = 3737$.

2 $P$ Test with unequal variance.
## TABLE 4
Odds ratios (and 95% CIs) for incident diabetes by quintile of plasma phospholipid fatty acid proportions, Melbourne Collaborative Cohort Study, 1990–1994.

<table>
<thead>
<tr>
<th>Fatty acid and model</th>
<th>Quintile 2*</th>
<th>Quintile 3</th>
<th>Quintile 4</th>
<th>Quintile 5</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>15:0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1*</td>
<td>0.73 (0.54, 0.99)</td>
<td>0.36 (0.25, 0.52)</td>
<td>0.32 (0.22, 0.47)</td>
<td>0.26 (0.17, 0.40)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Model 2</td>
<td>0.83 (0.60, 1.15)</td>
<td>0.46 (0.31, 0.68)</td>
<td>0.45 (0.30, 0.68)</td>
<td>0.40 (0.26, 0.63)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>16:0</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>1.14 (0.79, 1.65)</td>
<td>1.43 (0.99, 2.06)</td>
<td>1.37 (0.94, 2.00)</td>
<td>1.32 (0.89, 1.94)</td>
<td>0.106</td>
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<tr>
<td>Model 2</td>
<td>1.10 (0.74, 1.65)</td>
<td>1.44 (0.97, 2.13)</td>
<td>1.33 (0.89, 1.99)</td>
<td>1.27 (0.84, 1.92)</td>
<td>0.171</td>
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<tr>
<td>18:0</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>1.46 (0.87, 2.45)</td>
<td>1.99 (1.22, 3.32)</td>
<td>2.12 (1.32, 3.43)</td>
<td>4.14 (2.65, 6.49)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Model 2</td>
<td>1.42 (0.83, 2.45)</td>
<td>1.70 (1.02, 2.83)</td>
<td>1.34 (0.81, 2.23)</td>
<td>2.25 (1.39, 3.62)</td>
<td>&lt; 0.0001</td>
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</table>

SFA

<table>
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<tr>
<th>Fatty acid and model</th>
<th>Quintile 2*</th>
<th>Quintile 3</th>
<th>Quintile 4</th>
<th>Quintile 5</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
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<td>15:0</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Model 1*</td>
<td>1.47 (0.90, 2.42)</td>
<td>2.43 (1.53, 3.85)</td>
<td>3.48 (2.23, 5.42)</td>
<td>3.76 (2.43, 5.81)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Model 2</td>
<td>1.12 (0.66, 1.89)</td>
<td>1.62 (0.99, 2.63)</td>
<td>2.02 (1.26, 3.22)</td>
<td>1.88 (1.19, 2.99)</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

trans Fatty acids

<table>
<thead>
<tr>
<th>Fatty acid and model</th>
<th>Quintile 2*</th>
<th>Quintile 3</th>
<th>Quintile 4</th>
<th>Quintile 5</th>
<th>P for trend</th>
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<td>15:0</td>
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<td></td>
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<tr>
<td>Model 1*</td>
<td>0.69 (0.50, 0.95)</td>
<td>0.39 (0.27, 0.58)</td>
<td>0.38 (0.25, 0.57)</td>
<td>0.20 (0.12, 0.32)</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Model 1</td>
<td>0.81 (0.56, 1.18)</td>
<td>0.87 (0.60, 1.25)</td>
<td>0.70 (0.47, 1.02)</td>
<td>0.88 (0.60, 1.24)</td>
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<tr>
<td>Model 2</td>
<td>0.84 (0.56, 1.25)</td>
<td>0.89 (0.60, 1.33)</td>
<td>0.75 (0.50, 1.13)</td>
<td>0.93 (0.50, 1.13)</td>
<td>0.399</td>
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CLA

<table>
<thead>
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<th>Fatty acid and model</th>
<th>Quintile 2*</th>
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<th>Quintile 5</th>
<th>P for trend</th>
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<td>15:0</td>
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<tr>
<td>Model 1*</td>
<td>0.71 (0.50, 1.01)</td>
<td>0.92 (0.64, 1.33)</td>
<td>0.80 (0.54, 1.21)</td>
<td>0.84 (0.56, 1.27)</td>
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<tr>
<td>Model 2</td>
<td>0.63 (0.43, 0.92)</td>
<td>0.78 (0.52, 1.17)</td>
<td>0.69 (0.44, 1.06)</td>
<td>0.73 (0.47, 1.14)</td>
<td>0.267</td>
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MUFAs

<table>
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<th>Fatty acid and model</th>
<th>Quintile 2*</th>
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<th>Quintile 4</th>
<th>Quintile 5</th>
<th>P for trend</th>
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<td>15:0</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Model 1*</td>
<td>0.47 (0.34, 0.66)</td>
<td>0.53 (0.38, 0.74)</td>
<td>0.49 (0.34, 0.70)</td>
<td>0.22 (0.14, 0.36)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Model 2</td>
<td>0.50 (0.35, 0.71)</td>
<td>0.64 (0.45, 0.94)</td>
<td>0.65 (0.44, 0.94)</td>
<td>0.33 (0.20, 0.56)</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

PUFAs

<table>
<thead>
<tr>
<th>Fatty acid and model</th>
<th>Quintile 2*</th>
<th>Quintile 3</th>
<th>Quintile 4</th>
<th>Quintile 5</th>
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<td>15:0</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Model 1*</td>
<td>2.07 (1.09, 3.95)</td>
<td>2.69 (1.42, 5.04)</td>
<td>4.77 (2.66, 8.58)</td>
<td>9.65 (5.48, 16.97)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Model 2</td>
<td>1.75 (0.90, 3.39)</td>
<td>1.68 (0.88, 3.21)</td>
<td>2.93 (1.60, 5.39)</td>
<td>4.48 (2.48, 8.08)</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

1  n = 3737. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; CLA, conjugated linoleic acid; PUFA, polyunsaturated fatty acids.

2  Quintile 1 was the referent.

3  Odds ratios by logistic regression; model 1 was adjusted for age, sex, country of birth, family history of diabetes, physical activity, and alcohol intake.

4  Odds ratios by logistic regression; model 2 was adjusted for age, sex, country of birth, family history of diabetes, physical activity, alcohol intake, BMI, and waist-hip ratio.
Our findings for plasma phospholipid fatty acids are generally consistent with the results of previous prospective studies. Positive associations between palmitoleic, dihomo-γ-linolenic, and SFAs in cholesterol esters and diabetes incidence, and an inverse association for linoleic acid, were reported previously (1, 3). For SFAs in cholesterol esters and diabetes incidence, and an inverse association between plasma phospholipid pentadecanoic acid and diabetes incidence. Pentadecanoic acid is a marker of MUFA intake and increased insulin resistance (23) and diabetes (24, 25).

How can the opposite associations with risk of diabetes seen for diet and plasma phospholipid linoleic acid be reconciled (ie, how is it possible for cases to report higher intake but have lower plasma phospholipid proportions than controls)? Differential reporting of intake by those who subsequently developed diabetes would have meant that the slopes of the regression of plasma phospholipid linoleic acid on diet linoleic acid differed according to diabetes status at follow-up, but this was not the case. Thus, differential measurement error is an unlikely explanation. Non-differential measurement error of linoleic acid in both diet and plasma would have attenuated the associations, in which case the conflicting associations are likely to be greater than what we observed.

Our data showed clearly that for any level of dietary linoleic acid, cases had lower plasma phospholipid linoleic acid proportions than did controls, which suggests that some metabolic difference may exist in persons with pre-diabetes that explains the conflicting results. The relatively low proportions of plasma phospholipid linoleic acid in persons who went on to develop diabetes appeared to be balanced by higher proportions of some longer, less saturated metabolites of linoleic acid. There is evidence that Δ⁶ desaturase activity (which catalyzes the metabolism of 18:2n−6 to 18:3n−6) may be modified by insulin when insulin is low (3, 26). If Δ⁶ desaturase activity is higher at higher insulin concentrations (16), this could contribute to the relatively low proportion of linoleic acid and relatively high proportion of dihomo-γ-linolenic acid (via γ-linolenic acid) seen in persons who developed diabetes and who were likely to be relatively hyperinsulinemic (27, 28). We found no evidence that arachidonic acid, the next step in n−6 fatty acid metabolism after dihomo-γ-linolenic acid, was associated with diabetes incidence.

Although MUFA have tended to be considered healthy fats, and in the KANWU study had a beneficial effect on insulin sensitivity (20), positive associations were seen between dietary MUFA intake and type 2 diabetes in our and other studies (14, 29, 30). This may reflect the fact that MUFAs in many Western

<table>
<thead>
<tr>
<th>Fatty acid and model</th>
<th>Quintile 1 odds ratio (95% CI)</th>
<th>Quintile 2 odds ratio (95% CI)</th>
<th>Quintile 3 odds ratio (95% CI)</th>
<th>Quintile 4 odds ratio (95% CI)</th>
<th>Quintile 5 odds ratio (95% CI)</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:1n−7/16:0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>2.08 (1.33, 3.26)</td>
<td>2.32 (1.46, 3.67)</td>
<td>3.44 (2.19, 5.41)</td>
<td>6.70 (4.35, 10.32)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td>1.80 (1.13, 2.88)</td>
<td>1.89 (1.17, 3.04)</td>
<td>2.55 (1.59, 4.08)</td>
<td>4.12 (2.63, 6.45)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>18:1n−9/18:0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>0.76 (0.55, 1.05)</td>
<td>0.57 (0.40, 0.82)</td>
<td>0.55 (0.38, 0.79)</td>
<td>0.49 (0.34, 0.71)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td>0.87 (0.61, 1.25)</td>
<td>0.80 (0.55, 1.18)</td>
<td>0.71 (0.48, 1.05)</td>
<td>0.73 (0.49, 1.09)</td>
<td>0.076</td>
<td></td>
</tr>
<tr>
<td>20:3n−6/18:2n−6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>2.26 (1.07, 4.78)</td>
<td>3.46 (1.71, 7.02)</td>
<td>7.23 (3.71, 14.11)</td>
<td>12.02 (6.24, 23.17)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td>1.74 (0.81, 3.74)</td>
<td>2.35 (1.14, 4.84)</td>
<td>4.12 (2.07, 8.20)</td>
<td>5.39 (2.74, 10.62)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>20:4n−6/20:3n−6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>0.65 (0.48, 0.89)</td>
<td>0.52 (0.38, 0.73)</td>
<td>0.35 (0.24, 0.51)</td>
<td>0.24 (0.16, 0.37)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td>0.82 (0.59, 1.15)</td>
<td>0.75 (0.52, 1.07)</td>
<td>0.57 (0.38, 0.85)</td>
<td>0.46 (0.38, 0.85)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>22:6n−3/22:5n−3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>0.89 (0.60, 1.34)</td>
<td>1.25 (0.85, 1.82)</td>
<td>1.30 (0.89, 1.89)</td>
<td>1.12 (0.76, 1.66)</td>
<td>0.273</td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td>0.85 (0.55, 1.32)</td>
<td>1.30 (0.87, 1.95)</td>
<td>1.32 (0.88, 1.97)</td>
<td>1.02 (0.68, 1.55)</td>
<td>0.523</td>
<td></td>
</tr>
</tbody>
</table>

1 n = 3737.
2 Quintile 1 was the referent.
3 Odds ratios by logistic regression; model 1 was adjusted for age, sex, country of birth, family history of diabetes, physical activity, and alcohol intake.
4 Odds ratios by logistic regression; model 2 was adjusted for age, sex, country of birth, family history of diabetes, physical activity, alcohol intake, BMI, and waist-hip ratio.
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FATTY ACIDS AND DIABETES
TABLE 6
Odds ratios (and 95% CIs) for incident diabetes by quintile of dietary fatty acids, Melbourne Collaborative Cohort Study, 1990 –19941
Fatty acid and model
Total fat
Model 13
Model 24
15:0
Model 1
Model 2
16:0
Model 1
Model 2
18:0
Model 1
Model 2
SFA
Model 1
Model 2
trans Fatty acids
Model 1
Model 2
16:1nҀ7
Model 1
Model 2
18:1nҀ9
Model 1
Model 2
MUFA
Model 1
Model 2
18:2nҀ6
Model 1
Model 2
20:3nҀ6
Model 1
Model 2
20:4nҀ6
Model 1
Model 2
Total nҀ6
Model 1
Model 2
18:3nҀ3
Model 1
Model 2
20:5nҀ3
Model 1
Model 2
22:5nҀ3
Model 1
Model 2
22:6nҀ3
Model 1
Model 2
Total nҀ3
Model 1
Model 2
PUFA
Model 1
Model 2
nҀ6:nҀ3
Model 1
Model 2

Quintile 22

Quintile 3

Quintile 4

1.27 (0.85, 1.89)
1.26 (0.82, 1.93)

1.24 (0.841, 1.85)
1.15 (0.75, 1.75)

1.22 (0.81, 1.83)
1.05 (0.66, 1.57)

1.59 (1.08, 2.33)
1.12 (0.76, 1.73)

0.023
0.894

1.07 (0.76, 1.51)
1.22 (0.85, 1.76)

1.13 (0.79, 1.60)
1.13 (0.78, 1.65)

0.93 (0.64, 1.36)
1.06 (0.71, 1.58)

0.75 (0.64, 1.36)
0.82 (0.54, 1.25)

0.101
0.232

1.31 (0.88, 1.95)
1.35 (0.89, 2.06)

1.32 (0.89, 1.95)
1.16 (0.76, 1.78)

1.45 (0.98, 2.14)
1.24 (0.82, 1.90)

1.65 (1.12, 2.43)
1.22 (0.80, 1.85)

0.012
0.558

1.10 (0.75, 1.61)
1.11 (0.73, 1.67)

1.08 (0.74, 1.58)
0.98 (0.65, 1.49)

1.32 (0.90, 1.93)
1.01 (0.67, 1.53)

1.46 (1.00, 2.14)
1.23 (0.81, 1.85)

0.030
0.421

1.14 (0.78, 1.65)
1.12 (0.75, 1.68)

1.19 (0.82, 1.73)
1.11 (0.74, 1.66)

1.34 (0.92, 1.96)
1.11 (0.74, 1.67)

1.22 (0.83, 1.81)
1.04 (0.68, 1.58)

0.214
0.924

0.91 (0.62, 1.33)
0.94 (0.62, 1.42)

0.97 (0.66, 1.43)
0.91 (0.59, 1.38)

0.84 (0.57, 1.24)
0.75 (0.49, 1.14)

1.19 (0.83, 1.71)
0.97 (0.66, 1.44)

0.170
0.991

0.67 (0.44, 1.03)
0.57 (0.36, 0.90)

1.07 (0.73, 1.57)
0.83 (0.55, 1.26)

1.05 (0.72, 1.53)
0.87 (0.58, 1.30)

1.33 (0.92, 1.90
0.88 (0.60, 1.31)

0.014
0.715

1.05 (0.69, 1.60)
0.90 (0.571, .41)

1.23 (0.82, 1.85)
0.97 (0.62, 1.50)

1.27 (0.85, 1.91)
0.90 (0.58, 1.39)

1.51 (1.03, 2.23)
1.04 (0.69, 1.59)

0.017
0.698

1.09 (0.71, 1.67)
0.92 (0.58, 1.45)

1.20 (0.79, 1.82)
0.94 (0.60, 1.48)

1.54 (1.03, 2.30)
1.10 (0.71, 1.70)

1.52 (1.02, 2.26)
1.04 (0.68, 1.58)

0.012
0.629

1.25 (0.84, 1.87)
1.29 (0.84, 1.97)

1.30 (0.87, 1.94)
1.13 (0.74, 1.74)

1.67 (1.13, 2.47)
1.48 (0.97, 2.25)

1.77 (1.19, 2.64*)
1.41 (0.92, 2.17)

0.002
0.107

0.88 (0.60, 1.28)
0.83 (0.55, 1.24)

0.98 (0.68, 1.41)
0.88 (0.60, 1.30)

0.96 (0.67, 1.39)
0.80 (0.54, 1.19)

1.16 (0.81, 1.66)
0.92 (0.62, 1.35)

0.222
0.901

1.45 (0.98, 2.13)
1.36 (0.88, 2.06)

0.87 (0.57, 1.32)
0.81 (0.52, 1.27)

1.34 (0.92, 1.96)
1.16 (0.78, 1.75)

1.07 (0.73, 1.57)
0.81 (0.54, 1.23)

0.849
0.095

1.25 (0.83, 1.86)
1.27 (0.83, 1.85)

1.26 (0.84, 1.88)
1.10 (0.71, 1.68)

1.70 (1.14, 2.50)
1.49 (0.98, 2.27)

1.70 (1.21, 2.68)
1.42 (0.93, 2.18)

0.001
0.087

1.28 (0.86, 1.90)
1.07 (0.70, 1.64)

1.00 (0.66, 1.51)
0.89 (0.57, 1.38)

1.33 (0.90, 1.96)
1.01 (0.66, 1.55)

1.49 (1.02, 2.19)
1.14 (0.75, 1.73)

0.039
0.541

0.97 (0.67, 1.40)
1.01 (0.68, 1.49)

0.82 (0.56, 1.19)
0.73 (0.49, 1.10)

0.94 (0.67, 1.40)
0.86 (0.62, 1.34)

0.92 (0.67, 1.40)
0.68 (0.62, 1.34)

0.961
0.191

1.07 (0.74, 1.55)
1.10 (0.74, 1.62)

0.84 (0.57, 1.23)
0.76 (0.50, 1.15)

0.99 (0.69, 1.44)
0.97 (0.65, 1.45)

0.96 (0.69, 1.44)
0.81 (0.54, 1.21)

0.785
0.233

1.12 (0.77, 1.62)
1.12 (0.75, 1.67)

0.79 (0.54, 1.18)
0.69 (0.45, 1.05)

1.16 (0.81, 1.67)
1.07 (0.72, 1.57)

0.95 (0.69, 1.45)
0.77 (0.52, 1.16)

0.955
0.181

1.33 (0.89, 1.98)
1.10 (0.72, 1.69)

1.28 (0.86, 1.91)
1.06 (0.69, 1.63)

1.06 (0.71, 1.60)
0.88 (0.57, 1.36)

1.44 (0.97, 2.13)
0.97 (0.63, 1.48)

0.187
0.564

1.18 (0.79, 1.75)
1.18 (0.78, 1.81)

1.13 (0.75, 1.69)
0.91 (0.59, 1.41)

1.67 (1.13, 2.45)
1.46 (0.97, 2.21)

1.70 (1.14, 2.52)
1.29 (0.84, 1.97)

0.002
0.133

1.28 (0.89, 1.84)
1.33 (0.90, 1.96)

1.43 (0.99, 2.07)
1.42 (0.96, 2.12)

1.35 (0.91, 1.98)
1.43 (0.94, 2.18)

1.51 (1.02, 2.23)
1.56 (1.03, 2.36)

0.055
0.051

Quintile 5

P for trend

n ҃ 3737. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
Quintile 1 was the referent.
3
Odds ratios by logistic regression; model 1 was adjusted for age, sex, country of birth, family history of diabetes, physical activity, and alcohol intake.
4
Odds ratios by logistic regression; model 2 was adjusted for age, sex, country of birth, family history of diabetes, physical activity, alcohol intake, BMI,
and waist-hip ratio.
1
2


countries are derived largely from meat and hence are correlated with SFAs (4, 30) or that benefits relating to oleic acid, the principal dietary MUFA, are attributable to other components of olive oil or characteristics of a diet high in olive oil (31).

Both plasma phospholipid and dietary 16:1n-7 were positively associated with diabetes risk, as was the ratio of 16:1n-7 to 16:0, which reflects stearoyl-CoA desaturase activity (16). On the other hand, the ratio of 18:1n-9 to 18:0, which also reflects stearoyl-CoA desaturase activity, was inversely related with diabetes incidence. In Swedish men, only the ratio of 16:1 n-7 to 16:0, but not that of 18:1n-9 to 18:0, was associated with development of the metabolic syndrome over 20 y of follow-up (16). An increased ratio of 16:1n-7 to 16:0 has been observed in other insulin-resistant states, but it is not known whether the association is a causal one (32). Recent studies in mice suggest that a lack of stearoyl-CoA desaturase prevents dietary induced obesity and suggest this enzyme as a target for anti-obesity drugs (33).

Avoidance of saturated fats and widespread adoption of PUFA-rich fats and oils has led to relatively high intakes of n-6 fatty acids and ratios of n-6 to n-3 fatty acids of around 10:1 or higher in the United States and 9:1 in the MCCS. Such ratios are in contrast with the recommended ratio of <4:1, which is based on estimates from traditional diets (34). The ratio of n-6 to n-3 fatty acids may contribute to insulin resistance (17, 35) and a range of other health conditions (36). It may be important that existing recommendations to limit SFA (37) intake do not lead to adverse effects of n-6 fatty acid consumption. Further intervention studies with insulin resistance as the outcome may clarify the most appropriate fat to substitute for SFAs (38).

The inverse association we observed between plasma phospholipid trans fatty acids and diabetes conflicts with 2 studies suggesting that dietary intake of trans fats has adverse effects on insulin sensitivity and diabetes risk (4, 29). The Iowa Women’s Health Study, however, found an inverse association between trans fatty acid intake and diabetes (39), and the difficulty of measuring trans fatty acid intake as the result of changes in dietary composition over time in the United States has been noted (40). Plasma phospholipid concentrations of trans fatty acids appear to reflect dietary intake (41), but we did not observe this association in the MCCS, possibly because of the very low levels of both dietary and plasma phospholipid trans fatty acids (6).

The lower proportion of linoleic acid in plasma phospholipid of those who developed diabetes relative to those who did not, given the correlation between phospholipid and dietary linoleic acid, suggests that increasing linoleic acid intake could reduce diabetes risk. However, our analysis of dietary intakes does not support this. If there is something about the pre-diabetic state that causes differences in plasma phospholipid fatty acid composition, prospective biomarker studies of diabetes will require careful interpretation. Reduction in dietary saturated fat intake may reduce diabetes risk. However, it is not clear how our results regarding linoleic acid should be interpreted, and recommendations to limit SFA intake should take into account the possible importance of the n-6:n-3 PUFA ratio. More work is required to determine the most appropriate dietary substitute for SFAs.

FIGURE 1. Distribution of plasma phospholipid linoleic acid percentages by quintile (Q) of linoleic acid intake at baseline in persons who remained healthy (controls) or who developed diabetes at follow-up 4 y later (cases). In a model with plasma linoleic acid percentage as the dependent variable and dietary linoleic acid modeled as the median of quintiles, there was no interaction (P = 0.6) between diabetes status at follow-up and linoleic acid intake. The proportion of plasma linoleic acid was higher by 0.022 (95% CI: 0.020, 0.025) for each increment in dietary intake and lower by 0.018 (95% CI: 0.015, 0.021) in persons who developed diabetes than in those who did not.

The authors’ contributions were as follows—AMH: design of analysis, statistical analysis, writing of the manuscript; DRE: interpretation of results and revision of manuscript drafts; RAG: responsibility for conduct and supervision of plasma fatty acid analyses, input into data interpretation, and commenting on manuscript drafts; AJS: interpretation of results and commenting on manuscript drafts; MM: responsibility for conduct and supervision of plasma fatty acid analyses, input into data interpretation, and commenting on manuscript drafts; KO’D: design of original MCCS and commenting on manuscript drafts; GGG: design and conduct of original MCCS and responsible for grants used to fund fatty acid assays. None of the authors had a conflict of interest.

REFERENCES


Intake of fried foods is associated with obesity in the cohort of Spanish adults from the European Prospective Investigation into Cancer and Nutrition1–3

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ABSTRACT

Background: Consumption of fried food has been suggested to promote obesity, but this association has seldom been studied.

Objective: We aimed to assess the association of energy intake from fried food with general and central obesity in Spain, a Mediterranean country where frying with oil is a traditional cooking procedure.

Design: This was a cross-sectional study of 33 542 Spanish persons aged 29–69 y who were participating in the European Prospective Investigation into Cancer and Nutrition between 1992 and 1996. Dietary intake was assessed by a diet history questionnaire. Height, weight, and waist circumference were measured by trained interviewers. Analyses were performed with logistic regression and were adjusted for total energy intake and other confounders.

Results: The prevalence of general obesity [body mass index (in kg/m²) ≥ 30] was 27.6% in men and 27.7% in women. Respective figures for central obesity (waist circumference ≥ 102 cm in men and ≥ 88 cm in women) were 34.5% and 42.6%. The average proportion of energy intake from fried food was 15.6% in men and 12.6% in women. The adjusted odds ratios for general obesity in the highest versus the lowest quintile of fried food intake were 1.26 (95% CI: 1.09, 1.45; P for trend < 0.001) in men and 1.25 (1.11, 1.41; P for trend < 0.001) in women. The corresponding values for central obesity were 1.17 (1.02, 1.34; P for trend < 0.003) in men and 1.27 (1.13, 1.42; P for trend < 0.001) in women.

Conclusion: Fried food was positively associated with general and central obesity only among subjects in the highest quintile of energy intake from fried food. Am J Clin Nutr 2007;86:198–205.

KEY WORDS Fried food, general obesity, central obesity, olive oil, Spain

INTRODUCTION

In Spain and in other Mediterranean countries, frying with oil is a traditional cooking procedure. Frying with oil modifies the fatty acid composition of food, increases the energy density, and reduces the water content (1). Frying may also improve food palatability by making food crunchy and aromatic (1, 2). However, the consumption of fried food may promote obesity through greater fat intake (3) and energy density (4).

Few studies have assessed the relation between fried food and obesity. In US children and adolescents, eating fried food away from home was associated with a higher body mass index (BMI; in kg/m²) and with greater weight gain (5). In the United States, fried food eaten away from home is usually prepared by deep-frying, and it is considered a proxy indicator for fast food intake (6). In Mediterranean countries, however, fried food is consumed at home as frequently as away from home, and both pan-frying and deep-frying are commonly used (7). Other studies assessing the association of fried food with obesity have been limited by having no account of total energy intake (EI) (8), by including fried food along with other types of food (9), or by considering only a limited number of items, such as potato chips (8, 10, 11) or fried snacks (8, 12–18).

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The objective of this analysis was to assess the association of EI from fried food with general and central obesity in Spain. We aimed to accomplish this objective by using data from a large study with a detailed assessment of food intake and food preparation techniques.

**SUBJECTS AND METHODS**

**Study design**

We used baseline data from the Spanish cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC). The design and methods of the EPIC study have been described elsewhere (19, 20). Briefly, EPIC is a cohort study of 520 000 European men and women that assesses the role of diet, lifestyle, and genetic and environmental factors with respect to the risk of cancer and other chronic conditions. EPIC-Spain is composed of 41 440 participants aged 29–69 y who were recruited from 1992 through 1996 in 5 regions in Spain: Asturias, Gipuzkoa, and Navarra in the north and Granada and Murcia in the south.

Of 41 440 participants, we excluded 816 with poor quality of dietary reports (defined as a ratio of estimated to required EI below the 1st percentile or above the 99th percentile; 21); 3546 who declared that they had modified their diet in the previous year; 2553 with a self-reported diagnosis of angina, myocardial infarction, stroke, cancer, or diabetes; and 33 with a BMI < 18.5 and 374 with a BMI > 40. Subjects with a BMI > 40 were excluded because of a possible predominant role of genetic factors in morbid obesity (22). We also excluded 143 participants with missing values for weight, height, or waist circumference (WC); 236 with missing data on educational attainment; 17 with no data on smoking; and 180 women who lacked information on parity. As a result, the final database for analyses included 33 542 subjects (12 905 men and 20 637 women). For analyses of the type of oil used for frying, we further excluded 3322 participants (1946 men and 1376 women) who used mixed oils for frying and 172 (78 men and 94 women) with missing data for this variable. These analyses were thus conducted with 30 048 subjects (10 881 men and 19 167 women).

All participants gave written informed consent before enrollment. The study protocol was approved by the ethics committees of the International Agency for Research on Cancer (Lyon, France) and of the Bellvitge Hospital (Barcelona, Spain).

**Diet history**

Usual food consumption in the previous year was assessed with the use of a computerized diet history questionnaire (23, 24), previously validated in Spain (25) and administered by trained interviewers. The average length of the diet history interview was 41 min. Each subject was asked about his or her food consumption in a typical week of the previous 12 mo. The preparation, frequency of consumption per week, and usual portion size of each food item were recorded. Seasonal differences in the patterns of consumption and variations between weekdays and weekends were also taken into account. A total of 662 different foods were recorded for all study participants, including foods items consumed individually or as part of 203 recipes and regional dishes. For each food, participants selected the portion size from a book of photographs prepared specifically for the study. Dishes and recipes containing combinations of food were recorded and later broken down into their simple-food components by following the proportions and combinations reported by the subject or by standard compositions following previously defined criteria (25).

Information on cooking methods was collected as part of the diet history. For 28% of the foods recorded in the study, the participant stated the method of preparation. For the 71% of the recorded foods for which the cooking method was not specified during the interview, a dietitian assigned the cooking method according to standard recipes or regional dishes. The cooking method was unknown for 1.0% of the foods recorded. The same food may be cooked by several methods as part of a single recipe. For example, in the preparation of paella (a typical Spanish recipe), rice is both fried and boiled. Thus, we defined fried food as food for which frying was the only cooking method used. Fried food could be deep-fried or pan-fried, and it could be battered, crumbed, or sautéed. An absorption coefficient was used to estimate the absorption of fat during frying for each fried food (26). We also collected information on the type of oil used for dressing, cooking, and frying. To estimate EIs, we used a food-composition table specifically developed for EPIC that included information on all regional dishes consumed (27).

**Other variables**

Standing height, weight, and WC were measured by trained observers according to standardized procedures (28). Anthropometric measures were made when participants were wearing light clothes and no shoes and after they had emptied the bladder. Height and weight were measured with electronic scales that were calibrated on a regular schedule. WC was measured at the point of smallest circumference between the iliac crest and the lowest rib, after a normal expiration. In obese subjects without a natural waist, WC was measured at the midpoint between the iliac crest and the lowest rib. General obesity was defined as a BMI ≥ 30. Central obesity was defined as WC ≥ 102 cm in men and ≥ 88 cm in women (28).

Information on sociodemographic and lifestyle characteristics and reproductive history was obtained through interview. Physical activity was assessed by using a previously validated questionnaire (29) that included activity at work, during leisure time, and during household chores. For activity at work, each participant was asked to classify his or her occupation as sedentary, standing, manual work, heavy manual work, or nonworker. The following activities during leisure time were ascertained in the previous year: walking, riding a bicycle, gardening, do-it-yourself work, and participation in sports (30). Energy expenditure, during either leisure-time activity or at household chores, was obtained as the sum of the products of the time spent in each activity by its assigned metabolic equivalent (MET), and was expressed in METs·h/wk (31). Education was assessed as the highest completed educational level, which was classified into 5 categories from uncompleted primary school to completed university. Participants were grouped into 3 categories of consumption of any type of tobacco (current smoker, ex-smoker, or never smoker), and the number of cigarettes consumed was recorded for smokers. Data on parity were obtained by asking the women how many children they have had. Women were classified as being postmenopausal when >1 y had passed since their last menstrual cycle.
Statistical analysis

Participants were classified in sex-specific quintiles of EI from fried food. The association of fried food intake with obesity was modeled with mixed-effects logistic models, with the study center as a random effect to account for regional differences in food preparation. We estimated the odds ratios of obesity for each of the 4 upper quintiles of percentage of the EI from fried food compared with the lowest quintile. Linear trends in odds ratios were tested for by modeling quintiles of intake as a continuous variable. Separate analyses were done for general and central obesity and for men and women.

Logistic regression models were adjusted for age (in 5-y periods), physical activity at work (sedentary occupation, standing occupation, manual work, heavy manual work, and nonworker), leisure-time physical activity (METs·h/wk in quintiles), household activity (METs·h/wk in quintiles), education level (no formal education, primary studies, vocational training, secondary studies, and university studies), and total EI as a continuous variable. Analyses for women were further adjusted for parity, menopausal status, and use of hormone replacement therapy. We fitted an energy-adjusted model by using the multivariate nutrient density method. In these models (32), the association of EI from fried food with obesity is interpreted as the odds ratio for obese women associated with replacing a given percentage of energy from nonfried food with the same percentage of energy from fried food.

The association of the consumption of specific groups of fried food with general and central obesity was assessed with logistic regression models and after adjustment for the same variables as previously described. Reported P values are 2-tailed. Random-effects logistic analyses were performed with the glimmix macro (33) and SAS statistical software (version 8.2; SAS Institute, Cary, NC).

RESULTS

The prevalence of general obesity was 27.6% in men and 27.7% in women; the prevalence of central obesity was 34.5% and 42.6%, respectively. The mean ± SD proportion of EI from fried food was 15.6 ± 7.3% in men and 12.6 ± 6.7% in women. A total of 206 different fried foods were recorded at least once, and the oils used for frying were olive oil, virgin olive oil, sunflower oil, corn oil, soy oil, and seed oils (the specific seed oil consumed was not reported), and mixed oils.

Increased EI from fried food was associated with greater intake of energy from lipids in both sexes and with higher total EI in women (Table 1). Participants of both sexes who were in sedentary occupations and those with a university education had a lower intake of fried food than did those who were more active and had less than a university education. In men, former smokers had a lower consumption of fried food than did women; among women, those with lower fried food intake were more likely to be older and postmenopausal. All of these associations were significant (P < 0.01).

The prevalence of general and central obesity increased with increasing intake of energy from fried food (Table 2). The odds ratios for general obesity in the highest versus the lowest quintile of EI from fried food were 1.26 (95% CI: 1.09, 1.45; P for linear trend < 0.001) in men and 1.25 (1.11, 1.41; P for linear trend < 0.001) in women. Corresponding odds ratios for central obesity were 1.17 (1.02, 1.34; P for linear trend = 0.003) and 1.28 (1.15, 1.42; P for linear trend < 0.001), respectively.

Overall, 69.6% of men and 68.3% of women reported using olive oil (regular or virgin), and the rest of the participants reported using other oils (sunflower, corn, or soy). Subjects in the highest quintile of fried food intake were more likely than were those in the lowest quintile to have both general and central obesity regardless of the type of oil used for frying, although the associations were not significant for general or central obesity and consumption of other oils in women or for central obesity and consumption of olive oil in men (Table 3).

Fried meat, fish, potatoes, and eggs were the 4 groups of fried food most frequently consumed by study participants, with >75% of men and women consuming each of those groups of food. EI in men and women, respectively, ranged from to 2.0% and 1.5% for fried eggs to 5.0% and 3.6% for fried meat. Consumption of fried meat was positively associated with general obesity in men, and the intake of fried fish was associated with general obesity in women. The same pattern was observed for central obesity. In addition, consumption of fried egg was associated with central obesity in men (Figure 1).

DISCUSSION

In this large cross-sectional analysis of the EPIC-Spain cohort, fried food intake was positively associated with general and central obesity regardless of total EI only in the subjects in the highest quintile of EI from fried foods, corresponding to >21.7% of total calorie intake in men and >18.0% of that in women. This association was observed in both sexes and in participants who reported using olive oil or other types of oil for frying.

During frying, food is totally or partially immersed in oil that is heated above 180 °C. This process modifies both the foods and the frying medium. In contact with hot frying oil, food loses water, absorbs oil, and exchanges lipids with the frying oil. The amount of oil absorbed by frying food depends on the type of food and the frying conditions, but it may be >20% of its weight. French fries, for example, may absorb 19% of their weight as oil during frying (34). Fried food also undergoes pyrolytical decomposition in surface layers, which results in the formation of heterocyclic amines. Finally, frying food absorbs degradation products of the frying oil, such as polymers and polar compounds. These products have been associated with different types of cancer (35–40), endothelial dysfunction (41), and hypertension (42).

Several mechanisms may explain a positive association between fried food intake and obesity. Fried foods are crunchy, aromatic, highly palatable, and rich in fats. As a consequence, eating fried food ad libitum may result in higher absolute intake of foods with high energy density and low satiety index. The relatively low satiety index of fats (43) may be related to their low ability to stimulate insulin and leptin production (44). In addition, fat intake may stimulate food consumption beyond its effect on satiety. For instance, when lunch and snacks with different content of fat and carbohydrates but with similar effects on postmeal satiety were offered, subjects consuming the high-fat lunch and snacks had higher fat and EI over the whole day than did subjects consuming high-carbohydrate foods (45).

Fried food may also induce obesity by increasing food energy density. Energy density, palatability, and EI are highly related, and...
<table>
<thead>
<tr>
<th></th>
<th>Men (n = 12905)</th>
<th></th>
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<th>Women (n = 20637)</th>
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<th>P for trend</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Q1</td>
<td>Q3</td>
<td>Q5</td>
<td></td>
<td>Q1</td>
<td>Q3</td>
<td>Q5</td>
</tr>
<tr>
<td>Energy from fried food (%)</td>
<td>6.0 ± 2.0 * 2</td>
<td>15.1 ± 1.1</td>
<td>26.4 ± 4.4</td>
<td>&lt; 0.0001</td>
<td>4.4 ± 1.7</td>
<td>11.7 ± 1.0</td>
<td>22.8 ± 4.3</td>
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<td>Total energy (kcal/d)</td>
<td>2738.6 ± 738.7</td>
<td>2865.7 ± 682.0</td>
<td>2708.2 ± 658.8</td>
<td>0.48</td>
<td>1943.8 ± 589.9</td>
<td>2054.5 ± 545.3</td>
<td>1974.8 ± 484.2</td>
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<td>Energy from lipids (%)</td>
<td>33.8 ± 6.3</td>
<td>35.0 ± 5.8</td>
<td>36.5 ± 5.6</td>
<td>&lt; 0.0001</td>
<td>35.3 ± 6.7</td>
<td>36.6 ± 5.7</td>
<td>38.5 ± 5.8</td>
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<td>Age (y)</td>
<td>50.5 ± 7.6</td>
<td>50.2 ± 7.0</td>
<td>50.9 ± 6.9</td>
<td>0.051</td>
<td>48.8 ± 8.6</td>
<td>47.8 ± 8.2</td>
<td>47.5 ± 8.0</td>
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<td>Physical activity at work (%)</td>
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<td>Sedentary occupation</td>
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<td>33.6</td>
<td>30.3</td>
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<td>14.4</td>
<td>12.2</td>
<td>11.9</td>
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<td>34.7</td>
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<td>82.0</td>
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<td>Manual work</td>
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<td>22.7</td>
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<td>2.0</td>
<td>2.6</td>
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<td>Heavy manual work</td>
<td>6.5</td>
<td>4.7</td>
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<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
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<td>Nonworker</td>
<td>4.5</td>
<td>4.3</td>
<td>5.2</td>
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<td>2.3</td>
<td>2.2</td>
<td>3.4</td>
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<tr>
<td>Leisure-time physical activity (METs · h/wk)</td>
<td>33.9 ± 29.0</td>
<td>33.4 ± 28.8</td>
<td>31.7 ± 28.5</td>
<td>0.0014</td>
<td>25.8 ± 21.2</td>
<td>26.0 ± 19.8</td>
<td>27.3 ± 20.1</td>
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<td>Physical activity in household (METs · h/wk)</td>
<td>18.8 ± 23.1</td>
<td>18.1 ± 22.5</td>
<td>17.7 ± 21.9</td>
<td>0.0007</td>
<td>97.3 ± 44.0</td>
<td>101.2 ± 42.8</td>
<td>99.0 ± 44.4</td>
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<td>Educational level (%)</td>
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<td>No formal education</td>
<td>29.9</td>
<td>23.7</td>
<td>26.4</td>
<td>&lt; 0.0001 * 3</td>
<td>45.1</td>
<td>38.1</td>
<td>30.9</td>
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<td>Primary school</td>
<td>31.8</td>
<td>38.5</td>
<td>42.1</td>
<td></td>
<td>32.9</td>
<td>41.1</td>
<td>49.1</td>
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<td>Vocational training</td>
<td>8.9</td>
<td>14.3</td>
<td>15.3</td>
<td></td>
<td>4.8</td>
<td>5.7</td>
<td>6.1</td>
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<td>Secondary school</td>
<td>8.8</td>
<td>8.1</td>
<td>6.6</td>
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<td>5.6</td>
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<td>6.5</td>
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<tr>
<td>University</td>
<td>20.5</td>
<td>15.5</td>
<td>9.7</td>
<td></td>
<td>11.7</td>
<td>9.5</td>
<td>7.5</td>
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<tr>
<td>Smoking</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Former smoker (%)</td>
<td>34.9</td>
<td>27.4</td>
<td>26.1</td>
<td>&lt; 0.0001 * 3</td>
<td>10.2</td>
<td>9.8</td>
<td>9.5</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>33.4</td>
<td>32.6</td>
<td>27.0</td>
<td></td>
<td>18.2</td>
<td>19.1</td>
<td>21.9</td>
</tr>
<tr>
<td>Never smoker (%)</td>
<td>31.7</td>
<td>40.0</td>
<td>46.9</td>
<td></td>
<td>71.6</td>
<td>71.1</td>
<td>68.6</td>
</tr>
<tr>
<td>Cigarettes (no./d)</td>
<td>17.3 ± 11.3</td>
<td>16.8 ± 11.3</td>
<td>16.7 ± 11.2</td>
<td>0.046 * 7</td>
<td>12.8 ± 8.8</td>
<td>13.5 ± 9.1</td>
<td>13.7 ± 8.6</td>
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<td>Reproductive history</td>
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<tr>
<td>Parity</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>2.5 ± 1.56</td>
<td>2.5 ± 1.4</td>
<td>2.2 ± 1.3</td>
</tr>
<tr>
<td>Menopausal status (%)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>41.3</td>
<td>35.4</td>
<td>33.1</td>
</tr>
<tr>
<td>Hormone replacement treatment (%)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>9.2</td>
<td>8.0</td>
<td>9.7</td>
</tr>
</tbody>
</table>

1 Cutoffs for quintiles of energy intake from fried food were 9.0%, 13.3%, 17.1%, and 21.7% in men and 6.7%, 10.0%, 13.5%, and 18.0% in women. Linear regression or logistic regression models were used to obtain the P value for linear trend, by introducing the quintiles of fried food as a continuous variable.

2 SD (all such values).

3 Chi-square test.
energy density is a primary determinant of voluntary EI (46). Studies have shown that energy density is a better predictor of total EI than is fat content in both lean and obese females (47). Energy density may thus affect EI independent of macronutrient content or palatability (4).

Fat intake with fried foods could be also associated with obesity because fats are absorbed and metabolized more efficiently than are other macronutrients (3, 48, 49). Unlike those mechanisms described above, this type of potential mechanism does not involve greater EI. It has been argued that body weight may depend on macronutrient composition in addition to total EI (50–53) and that low-fat, high-protein, and high-complex carbohydrate diets should be recommended for obesity prevention.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
<th>Q5</th>
<th>P for linear trend</th>
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<tbody>
<tr>
<td><strong>General obesity</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Men (n = 12,905)</td>
<td></td>
<td></td>
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<tr>
<td>Prevalence</td>
<td>27.4</td>
<td>27.1</td>
<td>26.3</td>
<td>28.1</td>
<td>32.6</td>
<td></td>
</tr>
<tr>
<td>Odds ratio (95% CI)</td>
<td>1 (Ref)</td>
<td>0.99 (0.87, 1.12)</td>
<td>0.96 (0.84, 1.10)</td>
<td>1.03 (0.89, 1.19)</td>
<td>1.26 (1.09, 1.45)</td>
<td>0.0009</td>
</tr>
<tr>
<td>Women (n = 20,637)</td>
<td></td>
<td></td>
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<tr>
<td>Prevalence</td>
<td>25.8</td>
<td>25.7</td>
<td>25.3</td>
<td>26.8</td>
<td>29.3</td>
<td></td>
</tr>
<tr>
<td>Odds ratio (95% CI)</td>
<td>1 (Ref)</td>
<td>1.01 (0.91, 1.12)</td>
<td>1.01 (0.91, 1.12)</td>
<td>1.10 (0.99, 1.23)</td>
<td>1.25 (1.11, 1.41)</td>
<td>0.0002</td>
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<tr>
<td><strong>Central obesity</strong></td>
<td></td>
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<tr>
<td>Men (n = 12,905)</td>
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</tr>
<tr>
<td>Prevalence</td>
<td>36.0</td>
<td>34.3</td>
<td>34.6</td>
<td>37.3</td>
<td>40.1</td>
<td></td>
</tr>
<tr>
<td>Odds ratio (95% CI)</td>
<td>1 (Ref)</td>
<td>0.91 (0.81, 1.03)</td>
<td>0.94 (0.83, 1.07)</td>
<td>1.05 (0.91, 1.20)</td>
<td>1.17 (1.02, 1.34)</td>
<td>0.0025</td>
</tr>
<tr>
<td>Women (n = 20,637)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Prevalence</td>
<td>40.3</td>
<td>41.1</td>
<td>40.0</td>
<td>42.5</td>
<td>44.5</td>
<td></td>
</tr>
<tr>
<td>Odds ratio (95% CI)</td>
<td>1 (Ref)</td>
<td>1.05 (0.95, 1.16)</td>
<td>1.02 (0.92, 1.13)</td>
<td>1.14 (1.03, 1.27)</td>
<td>1.28 (1.15, 1.42)</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

2. Prevalence of obesity: adjusted for center (Asturias, Gipuzkoa, Granada, Murcia, or Navarra).
3. Waist circumference: ≥ 102 and ≥ 88 cm in men and women, respectively. Logistic regression models were adjusted for age (29–≤40, >40–≤45, >45–≤50, >50–≤55, >55–≤60, or >60 y), physical activity at work (sedentary occupation, standing occupation, manual work, heavy manual work, or nonworker), leisure-time physical activity (metabolic equivalents h/wk in quintiles), physical activity in household (metabolic equivalents h/wk in quintiles), smoking status (former smoker or current no. of cigarettes/d), educational level (no formal education, primary school, vocational training, secondary school, or university), energy intake, and center (Asturias, Gipuzkoa, Granada, Murcia, or Navarra). Center was modeled as a random effect. For women, the model was also adjusted for parity, menopausal status, and hormone replacement treatment. Cutoffs for quintiles of energy intake from fried food were 9.0%, 13.3%, 17.1%, and 21.7% in men and 6.7%, 10.0%, 13.5%, and 18.0% in women.
Intake of fried foods is associated with obesity

General obesity

Men
- Fried meat
- Fried fish
- Fried potatoes
- Fried eggs

Women
- Fried meat
- Fried fish
- Fried potatoes
- Fried eggs

Central obesity

Men
- Fried meat
- Fried fish
- Fried potatoes
- Fried eggs

Women
- Fried meat
- Fried fish
- Fried potatoes
- Fried eggs

FIGURE 1. Odds ratios and 95% CIs of general and central obesity in the highest quintile versus the lowest quintile of percentage energy intake of 4 specific groups of fried foods. General obesity: BMI ≥ 30; central obesity: waist circumference ≥ 102 and ≥ 88 cm in men and women, respectively.

Nutrition–Spain. Logistic regression models were adjusted for age (29 to 40, >40 to ≤45, >45 to ≤50, >50 to ≤55, >55 to ≤60, or >60 y), physical activity at work (sedentary occupation, standing occupation, manual work, heavy manual work, or nonworker), leisure-time physical activity (metabolic equivalents · h/wk in quintiles), household physical activity (metabolic equivalents · h/wk in quintiles), smoking status (former smokers or no. cigarettes/d for current smokers), educational level (no formal education, primary school, vocational training, secondary school, or university), energy intake, percentage of energy of other fried foods listed in the figure, and study center (Asturias, Gipuzkoa, Granada, Murcia, or Navarra). The center was modeled as a random effect. For women, the model was also adjusted for parity, postmenopausal status, and hormone replacement treatment. Cutoffs for the highest and lowest quintiles of percentage energy intake in men were 8.4% and 1.0% for fried meat, 3.9% and 0.7% for fried fish, 4.17% and 0.75% for fried potatoes, and 3.2% and 0.5% for fried eggs. Cutoffs for the highest and lowest quintiles of energy intake in women were 6.3% and 1.0% for fried meat, 3.9% and 0.7% for fried fish, 4.17% and 0.75% for fried potatoes, and 3.2% and 0.5% for fried eggs. Cutoffs for the highest and lowest quintiles of percentage energy intake of 4

This view, however, is controversial. Although short-term studies show a modest reduction in body weight with low fat diets, long-term studies suggest that total EI is much more important than macronutrient composition for body weight (54).

Because the analyses were adjusted for EI, mechanisms not involving a greater EI are the most likely explanation for our findings. However, given that the estimation of EI in epidemiologic studies is less than perfect, other mechanisms implicating higher EI, such as increased food consumption and energy density, cannot be ruled out as contributors to the association between fried food intake and obesity.

A priori, we expected that the association between fried food and obesity would be linear. In women, there is certain indication of linearity because the ORs tended to increase fairly monotonically from the lowest quintile of fried food consumption. Moreover, given that the association between fried food and obesity is only modest for the highest quintile, we were not surprised that such an association was not observed in the intermediate quintiles. Our results, however, do not permit a conclusion as to whether the association is graded or whether it represents a threshold.

In our study, the association of fried food intake with obesity was observed both in subjects who reported using olive oil for frying and in those using other oils. Olive oil is more resistant to oxidation than are other oils (55), and it has been considered the oil of choice for frying. Deep frying in olive oil may not adversely affect postprandial endothelial function (41). Furthermore, insulin resistance was lower in subjects using olive oil for cooking than in those using sunflower oil or mixed oils (56). However, our results do not support a differential association of olive oil used for frying with general or central obesity.

Because the association between fried food and obesity is only modest in the highest quintile of consumption, it is more likely that we would find associations for fried food as a whole than for any individual type of fried food. This situation is comparable to others in nutritional epidemiology: in that field of study, it is easier to detect associations between health and certain overall dietary patterns (eg, “prudent patterns” defined as a priori or Mediterranean diet scores defined as a posteriori) than to detect associations between health and the individual dietary components (57). Nevertheless, we have found associations in the expected direction for fried meat and fried eggs in men and for fried fish in women.

An important strength of our study was the availability of a detailed diet history, which allowed the ascertainment of a wide variety of commonly consumed foods and of food-preparation methods. Information on cooking methods was available for 99% of the foods recorded. Other strengths included the use of a large sample of free-living participants from diverse geographic areas in Spain and the collection of information on many potentially important covariates, such as total EI and physical activity.

Several potential limitations, however, should be considered in the interpretation of our findings. The cross-sectional design of our analysis did not allow us to establish a causal relationship between fried food intake and obesity. We cannot know whether a higher intake of fried food preceded the development of obesity or was a consequence of it. In addition, it is possible that obese subjects changed their food intake, including that of fried food, in an attempt to lose weight. Furthermore, we cannot exclude differential biases in the reporting of fried food intake in obese compared with nonobese participants. In a study of 38 healthy females, underreporting of the intake of fried foods and candy was greater in the obese than in the nonobese participants (58). Indeed, fried foods that are widely perceived as unhealthy, such as fried potatoes, had no relation with obesity in our study. We speculate that this difference may be due to a combination of changes in dietary habits by obese participants to avoid fried potatoes and to differential underreporting of these food items by obese participants. In the EPIC–Spain cohort, total EI was underestimated by 5.5% in obese males and by 17.5% in obese females according to the discrepancy between reported intake and anthropometric measurements (59). Unfortunately, no data are available on the extent of differential underreporting of different types of foods, including fried foods, among obese subjects.

Deep-frying, in which frying foods are totally immersed in oil, and pan-frying, in which frying foods are only partially immersed in oil, have different effects on the absorption of fat by the...
frying foods, depending on the food, the oil used, and the frying conditions (60, 61). Our study, however, could not distinguish between the 2 frying methods. As a final limitation, it was not possible to separate the effect of a specific food from the effect of the cooking method.

Despite these limitations, our study provides important observational evidence of a positive association between fried food intake and obesity, a major public health problem. The prevalence of obesity in Mediterranean countries is already very high. Of the 10 European countries participating in EPIC, Spain, Italy, and Greece had the highest prevalences of obesity (28). Indeed, the prevalence of obesity in our study is only slightly lower than that in the United States, although the prevalence of morbid obesity was lower than that in the United States (62). Because fried food contains potentially carcinogenic products of degradation, a potential effect of fried food on the risk of obesity would add to the rationale for recommending other methods of cooking when possible and for reducing or avoiding some foods when frying is the only possible cooking procedure (eg, fried snacks). Because frying in oil is a common and widely accepted form of cooking, confirmation of our findings in prospective studies and in randomized controlled trials should be a priority in public health nutrition.

The authors’ responsibilities were as follows—CAG: coordinator of the European Prospective Investigation of Cancer and Nutrition—Spain; PG-C and FR-A: the concept of the study and writing the draft of the manuscript; CAG, AB, MDI, CM, JRQ, and CN: provision of the original data and information on the respective populations and input on the study design and analysis; and all authors: interpretation of results and contributions to the writing of the manuscript. None of the authors had a personal or financial conflict of interest.

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Hardness (difficulty of chewing) of the habitual diet in relation to body mass index and waist circumference in free-living Japanese women aged 18–22 y1–3

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ABSTRACT
Background: Animal studies suggest the beneficial effect of hardness of diet on body weight and adiposity. No human studies have examined hardness of diet in relation to obesity.
Objective: We examined cross-sectional associations of hardness of the habitual diet with body mass index (BMI; in kg/m²) and waist circumference in free-living humans.
Design: Subjects were 454 female Japanese dietetic students aged 18–22 y. Dietary hardness was assessed as an estimate of masticatory muscle activity for the habitual diet (ie, the difficulty of chewing the food). The consumption of a total of 107 foods was estimated by means of a self-administered, comprehensive diet history questionnaire, and masticatory muscle activity during the ingestion of these foods was estimated according to published equations. Waist circumference was measured at the level of the umbilicus.
Results: Mean BMI was 21.4 (95% CI: 21.1, 21.6), and mean waist circumference was 73.6 (72.9, 74.3) cm. Mean dietary hardness was 178 (175, 181) mV · s/1000 kcal. Dietary hardness was not significantly associated with BMI. However, it was negatively associated with waist circumference (P for trend = 0.005). This association remained after adjustment not only for potential confounding factors (P for trend = 0.028) but also for BMI (P for trend = 0.002).
Conclusions: Whereas no association between dietary hardness and BMI was seen, increasing dietary hardness was associated with lower waist circumference even after adjustment for BMI in free-living young Japanese women. This finding could make innovative contributions to the literature and raise issues for future studies regarding diet and obesity. Am J Clin Nutr 2007;86:206–13.

KEY WORDS Hardness of diet, body mass index, waist circumference, Japanese, women, diet history questionnaire, epidemiology

INTRODUCTION
Because the human genome has hardly changed since the emergence of behaviorally modern humans ≈10 000 y ago, contemporary humans are still genetically adapted for the foods consumed by our remote ancestors (1–5). The dietary choices of that time would necessarily have been limited to minimally processed or unprocessed—and, often, uncooked—wild plant and animal foods (2). In contrast, the contemporary diet in affluent societies mainly consists of foods that could not have been regularly consumed before the development of agriculture, industrialization, and advanced technology such as food-processing procedures; these foods include dairy products, cereals, refined cereals, refined sugars, refined vegetable oils, fatty meats, salt, and combinations of these foods (3). The collision of our ancient genome with the new conditions of life in affluent nations, including the dietary qualities of recently introduced foods is considered to be the ultimate factor underlying diseases of civilization, including obesity (3–5). Given that probability, the differences between the ancient dietary patterns and those currently prevalent in industrialized countries appear to have important implications for the prevention and treatment of contemporary chronic diseases, including obesity. A dietary characteristic that would differ greatly between the ancient dietary patterns and the contemporary dietary patterns in developed societies is hardness of the diet, referred to hereafter as dietary hardness.

However, no human studies have examined with diligence the possible association between dietary hardness and diseases of civilization, such as obesity. In contrast, several studies in mice...
(6, 7) and rats (8, 9) suggested the beneficial effect of dietary hardness on obesity. In this preliminary study, we tried to examine hardness of the habitual diet in relation to body mass index (BMI; in kg/m²) and waist circumference (WC; in cm) among young free-living Japanese women. For this examination, we assessed dietary hardness by using an estimate of masticatory muscle activity for the habitual diet, obtained with data on the consumption of a total of 107 foods estimated by a self-administered comprehensive diet history questionnaire (DHQ) (10–12) and data on masticatory muscle activities during the ingestion of these foods estimated according to published equations (13).

SUBJECTS AND METHODS

Subjects

The present study was based on a multicenter nutritional survey conducted in February and March 2006 among female dietetics students from 10 institutions in Japan. All measurements at each institution were conducted according to the survey protocol. Briefly, staff at each institution explained an outline of the survey to potential subjects. Subjects who responded positively were then provided detailed written and oral explanations of the general purpose and procedure of the survey. A total of 474 women took part. For the present analysis, we selected 454 women who met the following 3 inclusion criteria: they were 18–22 y old (n = 467); were not currently receiving dietary counseling from a doctor or dietitian (n = 468); and had a reported energy intake in the range of 1000–3500 kcal/d (n = 467).

Written informed consent was obtained from each subject, and also from a parent for subjects aged <20 y. The study protocol was approved by the Ethics Committee of the Japanese National Institute of Health and Nutrition (of Japan).

Dietary assessment

Dietary habits during the preceding month were assessed by using a self-administered comprehensive DHQ (10–12). Responses to the DHQ, as well as those to a lifestyle questionnaire, were checked at least twice for completeness. When necessary, forms were reviewed with the subject to ensure the clarity of answers. The DHQ is a 16-page structured questionnaire that consists of 7 sections: general dietary behavior; major cooking methods; consumption frequency and amount of 6 alcoholic beverages; consumption frequency and semiquantitative portion size of 118 selected food and nonalcoholic beverage items; dietary supplements; consumption frequency and semiquantitative portion size of 19 cereals (rice, bread, and noodles); soup consumed with noodles, and miso (fermented soybean paste) soup; and open-ended items for foods consumed regularly (≥1 time/wk) but not appearing in the DHQ (10). The food and beverage items were selected as foods commonly consumed in Japan, mainly from a food list used in the National Nutrition Survey of Japan, and standard portion sizes were derived mainly from several books of recipes for Japanese dishes (10). Estimates of dietary intake for a total of 150 food and beverage items (including 5 seasonings), energy, and nutrients were calculated by using an ad hoc computer algorithm for the DHQ based on the Standard Tables of Food Composition in Japan (14). Information on dietary supplements and data from the open-ended questionnaire items were not used in the calculation of dietary intake (10). Detailed descriptions of the methods used to calculate dietary intake and the validity of the DHQ with respect to nutrients have been published elsewhere (10–12). Pearson’s correlation coefficients between the DHQ and 3-d estimated dietary records were 0.48 for energy and 0.48–0.55 for macronutrients in 47 women (10). In addition, Pearson’s correlation coefficient between the DHQ and 16-d weighed dietary records was 0.71 for dietary fiber in 92 women, and the mean value of Spearman’s correlation coefficients of food groups was 0.44 (range: 0.13–0.77; S Sasaki, unpublished observations, 2006).

Estimation of dietary hardness

In the present study, dietary hardness was assessed as estimated masticatory muscle activity needed for the habitual diet (ie, the difficulty of chewing the food in the diet). Whereas the habitual diet was assessed by DHQ (10–12) as described above, estimates of masticatory muscle activity for each food in the DHQ were obtained from equations published by Yanagisawa et al (13). Those authors measured the activities of 6 muscle regions (mV ∙ s) involved in mastication (right and left masseters and anterior and posterior temporalis) by using electromyography during the ingestion of the same volume (1.3 × 1.3 × 1.3 cm) of 16 selected foods with various physical properties by 20 healthy Japanese adults (10 men and 10 women) with a mean age of 21 y. They found that masticatory muscle activities (mV ∙ s/2.197 cm³) were highly correlated with the physical properties of foods (ie, firmness, cohesiveness, and strain) as measured with a texturometer (GTX-2; Zenken KK Inc, Chiba, Japan) and developed the following equations (13):

\[
\text{Masticatory muscle activity} = 0.6586 \times \ln(\text{firmness} \times \text{cohesiveness} \times \text{strain} \times 10) - 0.0307
\]

(1)

where \(R^2 = 0.89\);

\[
\text{Masticatory muscle activity} = 0.2718 \times \text{firmness} + 0.0335 \times \text{strain} - 0.0030
\]

(2)

where \(R^2 = 0.89\); or

\[
\text{Masticatory muscle activity} = 0.3081 \times \text{firmness} + 0.3300
\]

(3)

where \(R^2 = 0.81\).

Using the information on the physical properties of foods they had measured earlier with a texturometer (15), Yanagisawa et al (13) then estimated masticatory muscle activities for a total of 144 foods according to one of their equations, by using the available variables (ie, firmness, cohesiveness, and strain). They did not, however, cross-validate the equations to show their applicability (13). We therefore conducted a cross-evaluation by using data reported by Shiono et al (16). Those authors measured the activities of 4 muscle regions (mV ∙ s) involved in mastication (right and left masseters and anterior temporalis, but not posterior temporalis) by using electromyography during the ingestion of standard-sized bites (2.4–44.5 g) of 46 selected foods with various physical properties by 6 healthy Japanese adults (3 men and 3 women) aged 23–27 y. By careful direct matching, information on masticatory muscle activities
for a total of 18 foods was available from Shiono et al (16) and information on physical properties was available from Yanagisawa et al (15). Pearson’s correlation coefficient between masticatory muscle activities measured by Shiono et al (mV s/g food) (16) and those estimated by using physical property values as described by Yanagisawa et al (mV s/2.197 cm³ divided by 2.197, assuming that the density of all foods = 1) (13) was 0.88 among these 18 foods. This high correlation suggests the applicability of the equations developed by Yanagisawa et al, despite the differences in masticatory muscles measured and in the amounts of foods consumed in the studies of Yanagisawa et al (13) and Shiono et al (16).

We directly matched each food item on the DHQ (n = 150) (10–12) with foods for which information on masticatory muscle activities was available (n = 144) from Yanagisawa et al (13). During the calculation of dietary hardness, we excluded from the 150 food items on the DHQ beverages (22 items), soups (4 items), seasonings including fat and oil (16 items), and water (1 item). Foods for which masticatory muscle activities had not been determined (21 items) were assigned a value according to that of a comparable food. Because the physical properties (and hence the hardness, or difficulty of chewing) of vegetables are greatly influenced by cooking with heat (13), we took those influences into account as much as possible. For tomatoes and cucumbers, we used values for raw tomatoes and raw cucumbers, respectively, because these vegetables are usually consumed without heating in Japan. For cabbage, we used a weighted mean of a value for raw cabbage and that for boiled leafy vegetables (because of a lack of information on boiled cabbage), based on the ratio of the observed consumption (g/d) of raw cabbage to that of cabbage cooked with heat (ie, 4:6) in 92 women (S Sasaki, unpublished observations, 2006). For carrots, we used a weighted mean of a value for raw carrots and that for boiled carrots, based on the ratio of the observed consumption (g/d) of raw carrots to that of carrots cooked with heat (ie, 3:7) in 92 women (S Sasaki, unpublished observations, 2006). For other vegetables, we used values adjusted for cooking with heat, given that these foods are usually consumed after cooking with heat in Japan. Dietary hardness was calculated as the sum of the products of estimated masticatory activities (mV s/2.197 cm³) and the volume of food consumed (cm³/d) divided by 2.197. For the estimation of food volume, we simply converted weight in grams to volume in cubic centimeters for all of the foods, on the assumption that the density of all foods = 1. Because the crude value of dietary hardness was strongly correlated with energy intake (Pearson’s correlation coefficient = 0.75), the energy-adjusted value (mV s/1000 kcal) was used in the present study. Estimates of masticatory muscle activity for the 107 food items used to calculate dietary hardness are presented in Table 1. We could not investigate the validity of the DHQ against the 16-d dietary records (which we used to investigate the validity of other dietary variables, as described above) in assessing dietary hardness, because an insufficient number of foods (n = 144 items) with information on hardness (ie, masticatory muscle activity) (13) prevented the calculation of dietary hardness by the 16-d dietary records.

Anthropometric measurements

Body height was measured to the nearest 0.1 cm while the subjects were standing and not wearing shoes. Body weight was measured to the nearest 0.1 kg while the subjects were wearing lightweight indoor clothing. WC was measured to the nearest 0.1 cm at the level of the umbilicus. The measurement was taken at the end of a normal respiration while the subjects were standing erect and with the arms at the side and the feet together.

Other variables

In the lifestyle questionnaire, the subject reported her residential area, which was grouped into 1 of 3 regions: northern (Kanto and Tohoku), central (Tokai, Hokuriku, and Kinki), or southern (Kyushu and Chugoku) Japan. The residential areas were also grouped into 3 categories according to population size (city with population ≥ 1 million, city with population < 1 million, or town or village). Current smoking status (yes or no) and whether the subject was currently trying to lose weight (yes or no) were self-reported in the lifestyle questionnaire. Physical activity was computed as the average metabolic equivalent-hours [MET h/d (17)], on the basis of the frequency and duration of 5 different activities (sleeping, high- and moderate-intensity activities, walking, and sedentary activities) over the preceding month as reported in the lifestyle questionnaire. Rate of eating (slow, medium, or fast) was self-reported as part of the DHQ.

Statistical analysis

All statistical analyses were performed with SAS software (version 8.2; SAS Institute Inc, Cary, NC). With the use of the PROC GLM procedure, linear regression models were constructed to examine the association of dietary hardness with BMI and WC. For analyses, subjects were categorized into quintiles according to dietary hardness values (mV s/1000 kcal). Mean (± SE) values of BMI and WC were calculated by quintiles of dietary hardness with or without adjustment for potential confounding factors, including residential area, size of residential area, current smoking, currently trying to lose weight, physical activity (total MET h/d, continuous), rate of eating, and energy intake (kcal/d, continuous). In the analysis of WC, BMI (continuous) was also included as a confounding variable. We also conducted analyses with further adjustment for nutrient intakes, including protein (% of energy, continuous), fat (% of energy, continuous), and dietary fiber (g/1000 kcal, continuous). Because alcohol intake was extremely low (x̄ = 1.4 g/d), alcohol intake was not considered a confounding factor. We tested for linear trends with increasing levels of dietary hardness by assigning each participant the median value for the category and modeling this value as a continuous variable. All reported P values are 2-tailed, and P < 0.05 was considered significant.

RESULTS

Basic characteristics of the subjects are shown in Table 2. Mean BMI was 21.4 (95% CI: 21.1, 21.6), and mean WC was 73.6 (72.9, 74.3) cm. Mean dietary hardness was 178 (175, 181) mV s/1000 kcal (range: 101–289 mV s/1000 kcal). The top contributor to dietary hardness was well-milled rice (27.0%), and next were spaghetti (4.1%), pork (3.9%), green leafy vegetables (3.7%), and cabbage (3.4%), as shown in Table 1. Potential confounding factors are shown by quintile of dietary hardness in Table 3. There was a negative association between dietary hardness and rate of eating. Dietary hardness was negatively associated with energy and fat intakes and positively associated with protein and dietary fiber intakes.
### TABLE 1

Hardness of the 107 food items used in the present study

<table>
<thead>
<tr>
<th>Food item</th>
<th>Contribution to dietary hardness (mV·s/1000 kcal %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well-milled rice</td>
<td>225 27.04 ± 15.22 ± 26.18</td>
</tr>
<tr>
<td>Spaghetti</td>
<td>339 4.07 ± 3.78</td>
</tr>
<tr>
<td>Pork</td>
<td>236 3.87 ± 2.97</td>
</tr>
<tr>
<td>Green leafy vegetables</td>
<td>1701 3.65 ± 3.32</td>
</tr>
<tr>
<td>Cabbage</td>
<td>2546 3.44 ± 3.04</td>
</tr>
<tr>
<td>Chicken</td>
<td>351 2.85 ± 2.37</td>
</tr>
<tr>
<td>Eggs</td>
<td>135 2.41 ± 1.50</td>
</tr>
<tr>
<td>Beef</td>
<td>345 2.37 ± 2.39</td>
</tr>
<tr>
<td>Japanese noodles (buckwheat and Japanese wheat noodles)</td>
<td>278 2.37 ± 2.19</td>
</tr>
<tr>
<td>Chinese cabbage</td>
<td>2404 1.84 ± 2.10</td>
</tr>
<tr>
<td>Mushrooms</td>
<td>2560 1.80 ± 1.66</td>
</tr>
<tr>
<td>Instant noodles</td>
<td>236 1.74 ± 2.48</td>
</tr>
<tr>
<td>White bread</td>
<td>76 1.60 ± 1.60</td>
</tr>
<tr>
<td>Apples</td>
<td>664 1.49 ± 2.19</td>
</tr>
<tr>
<td>Carrots</td>
<td>857 1.45 ± 1.07</td>
</tr>
<tr>
<td>Brown rice</td>
<td>230 1.41 ± 5.91</td>
</tr>
<tr>
<td>Well-milled rice with germ</td>
<td>227 1.33 ± 6.26</td>
</tr>
<tr>
<td>Wakame seaweed</td>
<td>3265 1.32 ± 1.50</td>
</tr>
<tr>
<td>Salted pickles (excluding plums)</td>
<td>2625 1.21 ± 2.15</td>
</tr>
<tr>
<td>Chinese noodles</td>
<td>236 1.19 ± 2.13</td>
</tr>
<tr>
<td>Japanese bread with a sweet filling</td>
<td>70 1.16 ± 1.46</td>
</tr>
<tr>
<td>Ground beef and pork</td>
<td>198 1.12 ± 0.98</td>
</tr>
<tr>
<td>Broccoli(^1)</td>
<td>1360 1.11 ± 1.38</td>
</tr>
<tr>
<td>Pizza</td>
<td>209 1.11 ± 1.97</td>
</tr>
<tr>
<td>Lettuce</td>
<td>3971 1.05 ± 1.21</td>
</tr>
<tr>
<td>Onions</td>
<td>555 0.97 ± 0.75</td>
</tr>
<tr>
<td>Natto (fermented soybeans)</td>
<td>129 0.94 ± 1.11</td>
</tr>
<tr>
<td>Oily fish</td>
<td>143 0.92 ± 0.82</td>
</tr>
<tr>
<td>Cucumbers</td>
<td>3327 0.82 ± 1.15</td>
</tr>
<tr>
<td>Shrimp</td>
<td>711 0.79 ± 0.68</td>
</tr>
<tr>
<td>Squid and octopus</td>
<td>691 0.79 ± 0.87</td>
</tr>
<tr>
<td>Well-milled rice mixed with barley</td>
<td>259 0.77 ± 4.91</td>
</tr>
<tr>
<td>White meat fish</td>
<td>229 0.76 ± 0.65</td>
</tr>
<tr>
<td>Bean sprouts</td>
<td>2892 0.74 ± 0.86</td>
</tr>
<tr>
<td>Oranges</td>
<td>212 0.74 ± 1.18</td>
</tr>
<tr>
<td>Red meat fish</td>
<td>225 0.73 ± 0.65</td>
</tr>
<tr>
<td>Dried fish</td>
<td>265 0.70 ± 1.12</td>
</tr>
<tr>
<td>Green peppers</td>
<td>2417 0.65 ± 0.91</td>
</tr>
<tr>
<td>70%+Milled rice</td>
<td>225 0.63 ± 4.64</td>
</tr>
<tr>
<td>Cornflakes</td>
<td>170 0.60 ± 1.66</td>
</tr>
<tr>
<td>Tofu (soybean curd) products</td>
<td>266 0.59 ± 0.98</td>
</tr>
<tr>
<td>French fries</td>
<td>218 0.59 ± 0.80</td>
</tr>
<tr>
<td>Cheese</td>
<td>91 0.58 ± 0.90</td>
</tr>
<tr>
<td>Sweet potatoes, yams, and taro</td>
<td>175 0.52 ± 0.55</td>
</tr>
<tr>
<td>Japanese-style pancakes(^4)</td>
<td>112 0.50 ± 0.73</td>
</tr>
<tr>
<td>Small fish with bones</td>
<td>766 0.47 ± 0.66</td>
</tr>
<tr>
<td>Butter roll</td>
<td>80 0.46 ± 1.00</td>
</tr>
<tr>
<td>Bananas</td>
<td>165 0.45 ± 0.80</td>
</tr>
<tr>
<td>Ground fish meat products</td>
<td>327 0.42 ± 0.64</td>
</tr>
<tr>
<td>Cakes</td>
<td>50 0.41 ± 0.46</td>
</tr>
<tr>
<td>Burdock</td>
<td>578 0.40 ± 0.61</td>
</tr>
<tr>
<td>Croissant</td>
<td>46 0.38 ± 0.76</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>568 0.38 ± 0.42</td>
</tr>
<tr>
<td>Potatoes</td>
<td>101 0.37 ± 0.38</td>
</tr>
<tr>
<td>Rice crackers</td>
<td>112 0.36 ± 0.56</td>
</tr>
<tr>
<td>Ham and sausages</td>
<td>97 0.36 ± 0.39</td>
</tr>
<tr>
<td>Radishes</td>
<td>450 0.35 ± 0.30</td>
</tr>
<tr>
<td>Sweetened yogurt(^1)</td>
<td>78 0.32 ± 0.38</td>
</tr>
<tr>
<td>Konnyaku (devil’s tongue jelly)</td>
<td>6309 0.32 ± 0.53</td>
</tr>
<tr>
<td>Japanese sweets with azuki beans</td>
<td>138 0.31 ± 0.43</td>
</tr>
<tr>
<td>Half-milled rice</td>
<td>227 0.30 ± 2.70</td>
</tr>
<tr>
<td>Tofu</td>
<td>74 0.30 ± 0.26</td>
</tr>
<tr>
<td>Shellfish other than oysters</td>
<td>1042 0.29 ± 0.41</td>
</tr>
</tbody>
</table>

\(^1\) These 107 food items from the 150 items in the diet history questionnaire were used for the calculation of dietary hardness. The remaining 43 items not used consisted of 22 beverages [fruit juice (100%), other fruit juice, tomato juice, vegetable juice, beer, sake, shochu, shochu mixed with water or a carbonated beverage, whisky, wine, green and oolong tea, black tea, coffee, cocoa, lactic acid bacteria beverages, sugar-sweetened soft drinks, sugar-free soft drinks, nutritional supplement drinks, full-fat milk, low-fat milk, skim milk, and cream or creamer added to coffee], 4 soups (corn soup, Chinese soup, soup consumed with noodles, and water for miso soup), 16 seasonings including fat and oil (sugar for coffee and black tea, sugar used during cooking, butter, margarine, mayonnaise, salad dressing, fat-free salad dressing, oil used during cooking, miso as seasoning, miso for miso soup, ketchup, table salt, salt used during cooking, soy sauce, curry and roux in stew, and artificial sweeteners), and drinking water. Food items are listed in the descending order of their mean contribution to overall dietary hardness.

\(^2\) Based on the data for subjects in the present study (454 Japanese women aged 18–22 y).

\(^3\) SD (all such values).

\(^4\) These 21 food items were assigned the hardness value of a comparable food.
TABLE 2
Basic characteristics of 454 Japanese women aged 18–22 y

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>19.6 ± 1.0</td>
</tr>
<tr>
<td>Body height (cm)</td>
<td>158.1 ± 5.5</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>53.4 ± 8.1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.4 ± 3.0</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>73.6 ± 7.4</td>
</tr>
<tr>
<td>Area of residence [n (%)]</td>
<td></td>
</tr>
<tr>
<td>North (Kanto and Tohoku)</td>
<td>267 (59)</td>
</tr>
<tr>
<td>Central (Tokai, Hokuriku, and Kinki)</td>
<td>85 (19)</td>
</tr>
<tr>
<td>South (Kyushu and Chugoku)</td>
<td>102 (22)</td>
</tr>
<tr>
<td>Size of residential area [n (%)]</td>
<td></td>
</tr>
<tr>
<td>City with a population ≥1 million</td>
<td>80 (18)</td>
</tr>
<tr>
<td>City with a population &lt;1 million</td>
<td>334 (74)</td>
</tr>
<tr>
<td>Town or village</td>
<td>40 (9)</td>
</tr>
<tr>
<td>Currently smoking [n (%)]</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>441 (97)</td>
</tr>
<tr>
<td>Yes</td>
<td>13 (3)</td>
</tr>
<tr>
<td>Currently trying to lose weight [n (%)]</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>342 (75)</td>
</tr>
<tr>
<td>Yes</td>
<td>112 (25)</td>
</tr>
<tr>
<td>Physical activity (total metabolic equivalents · h/d)</td>
<td>34.1 ± 3.5</td>
</tr>
<tr>
<td>Rate of eating [n (%)]</td>
<td></td>
</tr>
<tr>
<td>Slow</td>
<td>140 (31)</td>
</tr>
<tr>
<td>Medium</td>
<td>144 (32)</td>
</tr>
<tr>
<td>Fast</td>
<td>170 (37)</td>
</tr>
<tr>
<td>Energy intake (kcal/d)</td>
<td>1761 ± 406</td>
</tr>
<tr>
<td>Protein intake (% of energy)</td>
<td>13.9 ± 1.9</td>
</tr>
<tr>
<td>Fat intake (% of energy)</td>
<td>29.5 ± 5.0</td>
</tr>
<tr>
<td>Carbohydrate intake (% of energy)</td>
<td>55.1 ± 5.8</td>
</tr>
<tr>
<td>Dietary fiber intake (g/1000 kcal)</td>
<td>7.1 ± 2.1</td>
</tr>
<tr>
<td>Dietary hardness (mV · s/1000 kcal)</td>
<td>178 ± 31</td>
</tr>
</tbody>
</table>

1 x ± SD (all such values).

Mean values of BMI and WC across quintiles of dietary hardness are shown in Table 4. Dietary hardness was not significantly associated with BMI, regardless of adjustment for potential confounding factors. Conversely, dietary hardness was significantly and negatively associated with WC (in model 1, the mean difference in WC between the lowest and highest quintiles of dietary hardness was −2.9 cm; P for trend = 0.005). The significant negative association between dietary hardness and WC remained after adjustment for potential confounding factors (in model 2, mean difference: −2.7 cm; P for trend = 0.028) and also BMI (in model 4, mean difference: −2.4 cm; P for trend = 0.002). This inverse association seemed mainly due to the composition of the diet, because it disappeared after further adjustment for dietary intake (models 3 and 5).

DISCUSSION

To our knowledge, this is the first study to examine dietary hardness in relation to BMI and WC in humans. We found that, whereas there was no association with BMI, dietary hardness was negatively associated with WC even after adjustment for BMI in free-living young Japanese women. No human studies have examined the association between dietary hardness and obesity, but several animal studies have suggested the beneficial effect of a hard diet on obesity. Mice fed a hard diet from age 4 wk had a significantly lower body weight at age 36 wk than did mice fed a normal diet (6). In addition, body-weight gain from 4 to 9 wk of age was significantly smaller in male (but not female) mice fed a hard diet than in those fed a soft diet (7), and body-weight gain at age 6 wk was significantly smaller in rats fed a hard diet from age 1 wk than in those fed a soft diet (8). Furthermore, rats fed a hard diet from age 4–26 wk had significantly lower body weight and abdominal white adipose tissue than did those fed a soft diet (9).

TABLE 3
Selected characteristics of 454 Japanese women aged 18–22 y according to quintile (Q) of dietary hardness

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Q1 (n = 90)</th>
<th>Q2 (n = 91)</th>
<th>Q3 (n = 91)</th>
<th>Q4 (n = 91)</th>
<th>Q5 (n = 91)</th>
<th>P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dietary hardness (mV · s/1000 kcal)</td>
<td>137 ± 13</td>
<td>161 ± 5</td>
<td>176 ± 5</td>
<td>193 ± 6</td>
<td>223 ± 19</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Area of residence (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td>North (Kanto and Tohoku)</td>
<td>72</td>
<td>68</td>
<td>57</td>
<td>54</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Central (Tokai, Hokuriku, and Kinki)</td>
<td>17</td>
<td>20</td>
<td>14</td>
<td>15</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>South (Kyushu and Chugoku)</td>
<td>11</td>
<td>12</td>
<td>29</td>
<td>31</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Size of residential area (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.68</td>
</tr>
<tr>
<td>City with a population ≥1 million</td>
<td>18</td>
<td>16</td>
<td>19</td>
<td>24</td>
<td>11</td>
<td>0.42</td>
</tr>
<tr>
<td>City with a population &lt;1 million</td>
<td>79</td>
<td>78</td>
<td>68</td>
<td>66</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Town or village</td>
<td>3</td>
<td>5</td>
<td>13</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>1</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0.68</td>
</tr>
<tr>
<td>Subjects currently trying to lose weight (%)</td>
<td>26</td>
<td>29</td>
<td>21</td>
<td>29</td>
<td>20</td>
<td>0.21</td>
</tr>
<tr>
<td>Physical activity (total metabolic equivalents · h/d)</td>
<td>34.7 ± 5.2</td>
<td>33.8 ± 2.7</td>
<td>33.6 ± 2.4</td>
<td>34.1 ± 3.0</td>
<td>34.1 ± 3.3</td>
<td>0.51</td>
</tr>
<tr>
<td>Rate of eating (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>Slow</td>
<td>32</td>
<td>23</td>
<td>27</td>
<td>37</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>27</td>
<td>31</td>
<td>32</td>
<td>32</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Fast</td>
<td>41</td>
<td>46</td>
<td>41</td>
<td>31</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Energy intake (kcal/d)</td>
<td>1885 ± 419</td>
<td>1770 ± 410</td>
<td>1782 ± 375</td>
<td>1665 ± 394</td>
<td>1704 ± 403</td>
<td>0.0006</td>
</tr>
<tr>
<td>Protein intake (% of energy)</td>
<td>13.1 ± 1.6</td>
<td>13.4 ± 1.7</td>
<td>13.8 ± 1.6</td>
<td>14.1 ± 1.9</td>
<td>15.0 ± 1.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fat intake (% of energy)</td>
<td>31.0 ± 4.5</td>
<td>30.3 ± 5.6</td>
<td>29.8 ± 4.9</td>
<td>27.7 ± 5.0</td>
<td>28.6 ± 4.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Dietary fiber intake (g/1000 kcal)</td>
<td>6.0 ± 1.2</td>
<td>6.3 ± 1.2</td>
<td>6.6 ± 1.5</td>
<td>7.1 ± 1.8</td>
<td>9.5 ± 2.4</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

1 For continuous variables, a linear trend test was used with the median value in each quintile as a continuous variable in linear regression; a Mantel-Haenszel chi-square test was used for categorical variables.
2 x ± SD (all such values).
including a high intake of dietary fiber (18–21) and a low intake associated with healthier dietary patterns, including lower energy and associated with dietary composition in the diet of free-living while dietary composition remains constant, dietary hardness is whereas dietary hardness can freely be changed in animal models should be examined and interpreted with caution, because, hardness with obesity is independent of dietary composition findings from animal studies, because dietary hardness had a association between dietary hardness and WC was not independent of 

We do not know why we found unexpected null association with BMI (but found the expected inverse association with WC). Several limitations of the present study, such as the narrow range of BMIs in the subjects, the study’s cross-sectional design, and the use of a new and as yet unestablished method for assessing dietary hardness, may at least partly explain the null finding on BMI. Alternatively, the difference in abdominal white adipose tissue was much larger (22%) than that in body weight (6%) between rats fed a soft and those fed a hard diet (9), which may suggest that dietary hardness affects abdominal obesity (eg, WC) more strongly than it affects overall obesity (eg, BMI).

The negative association between dietary hardness and WC was independent of energy intake. Less body weight gain with a hard diet was related to decreased food intake in a study of rats (8). Conversely, the effect of dietary hardness on obesity was independent of the amount of foods consumed in other studies of mice (6) and rats (9), which may be due to increased thermogenesis (9) or unknown mechanisms. However, the negative association between dietary hardness and WC was not independent of diet composition, because that association disappeared after control for dietary composition. This finding is not consistent with findings from animal studies, because dietary hardness had a beneficial effect on obesity independent of diet composition (6–9). However, the question of whether the association of dietary hardness with obesity is independent of dietary composition should be examined and interpreted with caution, because, whereas dietary hardness can freely be changed in animal models while dietary composition remains constant, dietary hardness is associated with dietary composition in the diet of free-living humans. In the present study, greater dietary hardness was associated with healthier dietary patterns, including lower energy and fat and higher protein and dietary fiber. Several human studies have supported the favorable effects of healthy dietary patterns, including a high intake of dietary fiber (18–21) and a low intake of dietary fat (18, 19), on WC, which does not conflict with our finding.

Several limitations of the present study should be acknowledged. First, our subjects were selected female dietetics students, not a random sample of Japanese women, and the exact response rate was unknown because of our recruitment procedure; these elements of the design may produce recruitment bias. Thus, it may be that our results cannot be extrapolated to the general Japanese population.

Second, because this was a cross-sectional study, reverse causation may have occurred. However, it is unlikely that subjects with a large WC would intentionally change the hardness of their diet as a result of an increase in WC, because the notion that dietary hardness is associated with a measure of obesity is not well known. Furthermore, adjustment for intentional dietary change within the preceding year (yes or no), assessed as part of the DHQ, did not materially change the present results (data not shown). It is therefore reasonable to consider that our findings are not due to reverse causation.

Third, our DHQ was not designed specifically to measure dietary hardness, and the validity of the DHQ with respect to dietary hardness was unknown. The satisfactory validity of the DHQ for a wide range of nutrients and foods (10–12; S Sasaki, unpublished observations, 2006), however, may provide some reassurance. In addition, the DHQ may not adjust sufficiently for cooking methods in the calculation of dietary hardness. Our mean estimate of dietary hardness [crude \( \bar{x} \) (±SD): 312 ± 82 mV · s/d; range: 140–647 mV · s/d] was higher than that assessed by 3-d dietary records in a group of 140 women aged 18–23 y (267 ± 69 mV · s/d; 109–523 mV · s/d) (22), although the estimation of dietary hardness by using dietary records would be less reliable because the database of hardness (ie, masticatory muscle activity) is limited to a few food items (13). Moreover, we simply converted weight in grams to weight in

| TABLE 4 |
| -------------- | -------------- | -------------- | -------------- | -------------- | -------------- | -------------- | -------------- |
| BMI and waist circumference according to quintile (Q) of dietary hardness in 454 Japanese women aged 18–22 y | Q1 (n = 90) | Q2 (n = 91) | Q3 (n = 91) | Q4 (n = 91) | Q5 (n = 91) | P for trend\(^2\) |
| Dietary hardness (mV · s/1000 kcal) | 142 (101–152)\(^2\) | 163 (153–167) | 176 (168–183) | 192 (184–204) | 216 (205–289) | 
| BMI (kg/m\(^2\)) | 
| Model 1\(^5\) | 21.4 ± 0.3\(^4\) | 21.4 ± 0.3 | 21.4 ± 0.3 | 21.4 ± 0.3 | 21.1 ± 0.3 | 0.47 |
| Model 2\(^5\) | 21.3 ± 0.3 | 21.5 ± 0.3 | 21.3 ± 0.3 | 21.4 ± 0.3 | 21.2 ± 0.3 | 0.73 |
| Model 3\(^5\) | 21.1 ± 0.3 | 21.4 ± 0.3 | 21.2 ± 0.3 | 21.3 ± 0.3 | 21.7 ± 0.4 | 0.38 |
| Waist circumference (cm) | 
| Model 1 | 75.0 ± 0.8 | 74.4 ± 0.8 | 73.0 ± 0.8 | 73.8 ± 0.8 | 71.9 ± 0.8 | 0.005 |
| Model 2 | 74.9 ± 0.8 | 74.0 ± 0.8 | 73.1 ± 0.8 | 73.9 ± 0.8 | 72.2 ± 0.8 | 0.028 |
| Model 3 | 74.3 ± 0.8 | 73.7 ± 0.8 | 72.9 ± 0.8 | 73.7 ± 0.8 | 73.6 ± 0.9 | 0.63 |
| Model 4\(^5\) | 74.9 ± 0.5 | 73.7 ± 0.5 | 73.1 ± 0.5 | 73.8 ± 0.5 | 72.5 ± 0.5 | 0.002 |
| Model 5\(^5\) | 74.8 ± 0.5 | 73.6 ± 0.5 | 73.1 ± 0.5 | 73.8 ± 0.5 | 72.9 ± 0.6 | 0.063 |

1 A linear trend test was used with the median value in each quintile as a continuous variable in linear regression.
2 Median; range in parentheses (all such values).
3 Crude model.
4 ± SE (all such values).
5 Adjusted for residential area (Kanto and Tohoku in the north; Tokai, Hokuriku, and Kinki in the central area; or Kyushu and Chugoku in the south), size of residential area (city with a population ≥1 million, city with a population <1 million, or town or village), current smoking (yes or no), currently trying to lose weight (yes or no), physical activity (total metabolic equivalents h/d, continuous), rate of eating (slow, medium, or fast), and energy intake (kcal/d, continuous).
6 Adjusted for variables used in model 2, protein and fat intake (% of energy, continuous), and dietary fiber intake (g/1000 kcal, continuous).
7 Adjusted for variables used in model 2 and BMI (in kg/m\(^2\), continuous).
8 Adjusted for variables used in model 3 and BMI (in kg/m\(^2\), continuous).
cubic centimeters for all foods, assuming that the density of all foods = 1, even though for some foods that are high in air content (eg, snack foods), weight and volume are not directly proportional (23). Nevertheless, foods making the greatest contribution to dietary hardness in the present study did not seem to have this disproportional relation between weight and volume (see Table 1). Because the procedure we used provides only an approximation of the actual hardness of habitual diet, the results of the present study should be interpreted with great caution. Nevertheless, our findings should provide valuable insights into this poorly explored research issue.

Furthermore, misreporting of food intake, particularly by overweight persons, is a serious problem in self-reported dietary assessment methods (24). Consistent misreporting across all types of foods likely has little influence on energy-adjusted dietary hardness values (25), but studies indicate that overweight persons may selectively underreport their intakes of fatty or sugary foods (26, 27), which could cause dietary hardness estimations to be higher than actual values. In the present study, the potential shared error created by underreporting of dietary measures by subjects with a high BMI (and WC) would likely have weakened the associations of dietary hardness with measures of obesity and could possibly have led to a null finding; this possibility may at least partly explain the lack of association with BMI. Nonetheless, we did find a significant negative association with WC.

Finally, although we attempted to adjust for a wide range of potential confounding variables, we could not rule out residual confounding. Physical activity in particular was assessed relatively roughly from only 5 different activities, a number that may not have been sufficient. In addition, whereas dental status has an influence on food and nutrient intakes and on obesity (28–30), particularly in older persons, we unfortunately had no information on the subjects’ dental status, which could confound the present results for young women. Although impaired dental status may be less pervasive in young than in elderly populations, and although the percentage of subjects in a similar population (3828 Japanese female dietetics students aged 18–20 y) who had been diagnosed by a dentist as having a dental disease was relatively small (8%) (S Sasaki, unpublished observations, 2007), further research on dietary hardness and health should take the subjects’ dental status into account.

In conclusion, the results of the present study showed that, whereas there was no association between dietary hardness and BMI, dietary hardness was a significant independent determinant of WC in a group of free-living young Japanese women. Because these observations are generally consistent with the results of several animal studies (6–9), the present findings could make innovative contributions to the literature and raise issues for future studies on diet and obesity. However, because this is a preliminary study with a novel, as yet unestablished method of assessing dietary hardness, the results should be interpreted with great caution; nevertheless, applications of the method of assessing dietary hardness to other similar datasets would be of some interest. To better understand the influence of dietary hardness on obesity, further observational and intervention studies are clearly needed. To conduct such investigations, it is urgent to develop a database of values for a variable indicating hardness (eg, masticatory muscle activity) of various food items.

We thank Yukie Yanagisawa (Wayo Women’s University) for technical advice regarding the estimation of dietary hardness.

The author’s responsibilities were as follows—KMurakami: contributed to the concept and design of the study, the study protocol, data management, and coordinated the field work, calculated the dietary hardness, analyzed and interpreted the data, and wrote the manuscript; SS: the concept and design of the study, the study protocol, and data management, and contributed to the writing and editing of the manuscript; YT: the writing and editing of the manuscript; KU: the concept and design of the study, the study protocol, and data collection; MY, HH, TJ, JO, KB, KO, TK, KMuramatsu, and MF: data collection. All authors contributed to the preparation of the manuscript and approved the final version submitted for publication. None of the authors had any personal or financial conflict of interest.

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Plasma pyridoxal-5-phosphate and future risk of myocardial infarction in the European Prospective Investigation into Cancer and Nutrition Potsdam cohort\textsuperscript{1–3}

Jutta Dierkes, Cornelia Weikert, Kerstin Klipstein-Grobusch, Sabine Westphal, Claus Luley, Matthias Möhlig, Joachim Spranger, and Heiner Boeing

ABSTRACT

Background: Retrospective studies indicate that low concentrations of plasma pyridoxal-5-phosphate (PLP) are associated with cardiovascular events; however, few prospective studies of this issue have been conducted.

Objective: We therefore investigated whether PLP concentrations are independently associated with myocardial infarction (MI) in the European Investigation into Cancer and Nutrition (EPIC) Potsdam Study.

Design: After exclusion of prevalent MI or stroke, incident cases of MI were identified among 26 761 participants (aged 35–65 y at baseline). The current analysis is based on a nested case-cohort study consisting of a control group of 810 subjects without MI or stroke at baseline and a case group of 148 subjects who had an MI during a mean follow-up period of 6.0 ± 1.5 y. Cox proportional hazard models were used to evaluate the association between plasma PLP and risk of MI.

Results: In the age- and sex-adjusted analysis, subjects in the highest quintile of PLP had a significantly reduced risk of MI (hazard ratio: 0.50; 95% CI: 0.29, 0.83). Adjustment for either low-grade inflammation or smoking diminished this association. When both low-grade inflammation and smoking were adjusted for, the association was abolished. In addition, adjustment for established risk factors also abolished the association between PLP and risk of MI.

Conclusion: These findings from a prospective German cohort study suggest that PLP is not independently associated with risk of MI. 


KEY WORDS    Cardiovascular disease, epidemiology, vitamin B-6, inflammation, smoking

INTRODUCTION

There is increasing interest in B vitamins as risk factors for cardiovascular disease (CVD) because of the recognition of homocysteine as a potential risk factor for CVD \textsuperscript{(1)}, and it has been recognized that subnormal, but not necessarily deficient, concentrations of vitamins can be associated with an increased risk of CVD \textsuperscript{(2)}. Of the vitamins of interest, vitamin B-6 has received much attention because of its involvement in homocysteine metabolism, polyunsaturated fatty acid and lipid metabolism, and protein metabolism. Furthermore, vitamin B-6 was shown to inhibit platelet aggregation and endothelial cell proliferation, which suggests that it has an antithrombotic effect \textsuperscript{(3)}. Epidemiologic evidence for a potential protective action of vitamin B-6 first emerged from small studies in patients after a myocardial infarction (MI) \textsuperscript{(4)} and in mostly retrospective case-control studies involving patients with coronary, cerebral, or peripheral atherosclerotic disease \textsuperscript{(5–11)}. In most of those retrospective studies an association was found between low plasma pyridoxal-5-phosphate (PLP) and CVD. In addition, 2 corresponding prospective studies were published: one involved men participating in the Physicians’ Health Study \textsuperscript{(12)}, the other men and women of the Atherosclerosis Risk Factors in the Community (ARIC) study \textsuperscript{(13)}. These prospective studies reported divergent results for the association between PLP and CVD. Although in the Physicians’ Health Study the association was no longer significant after adjustment for established CVD risk factors, in the ARIC study multiple adjustment did not alter the results. Regardless of the study design, all studies so far investigated the risk of CVD associated with the plasma PLP concentration as a measure of the nutritional status of vitamin B-6. Subsequently, the interpretation of low plasma PLP became more difficult, because low-grade inflammation was found to be associated with low plasma PLP.

Friso et al \textsuperscript{(14)} showed that in the Framingham cohort chronic inflammation modulated plasma PLP concentrations. Despite similar vitamin B-6 intake, the plasma PLP concentrations were significantly lower in subjects with C-reactive protein (CRP) > 6 mg/L than subjects with CRP concentrations < 6 mg/L. This observation was confirmed in healthy elderly subjects in the United Kingdom \textsuperscript{(15)}, patients with rheumatoid arthritis \textsuperscript{(16)},...
inflammatory bowel disease (17), and type 2 diabetes (18). During the past decade the view on chronic inflammation as a cardiovascular disease risk factor changed considerably because of the discovery that chronic low-grade chronic inflammation is present in patients at high risk of CVD or patients with CVD (19, 20). The diagnosis of chronic low-grade inflammation became only possible after the introduction of measurement of highly sensitive CRP (hsCRP) as a sensitive laboratory method (21, 22). These findings question in particular the results of the retrospective studies on low plasma PLP and risk of CVD. So far, neither of the prospective studies and only 2 of the retrospective studies were adjusted for hsCRP (10, 11). Although Prisco et al (10) observed a significant association between low plasma PLP and CVD both before and after adjustment for CRP in patients with coronary artery disease, Dierkes et al (11) reported that the association disappeared completely after adjustment for hsCRP in a study involving women with prior MI or acute coronary syndrome.

There is therefore a need for prospective studies on the association between plasma PLP and cardiovascular disease risk that would take first markers of inflammation into account in the statistical procedure, and, second, investigate the associations of plasma PLP with other cardiovascular disease risk factors. In this analysis, we present data from the European Prospective Investigation into Cancer and Nutrition (EPIC) Potsdam cohort in Germany, with respect to the association of plasma PLP and future risk of MI.

SUBJECTS AND METHODS

Study population

The EPIC Potsdam cohort was recruited between August 1994 and September 1998 as part of the multicenter EPIC study. Details of this cohort as a part of the German study population were published elsewhere (23). Briefly, women aged 35–65 y and men aged 40–65 y were recruited at random from the general population. In total, 27 548 subjects (16 644 women and 10 904 men) underwent the baseline examination, including standardized blood pressure measurements, measurements of weight and height, self-administered questionnaires on diet and lifestyle, computer-guided interviews, and blood sampling. The study was approved by the Ethics Committee of Federal State Brandenburg, Germany, and written informed consent was obtained from all participants.

After the exclusion of subjects with a history of MI and stroke at baseline, we identified 156 participants who had a MI during follow-up until 30 April 2004. For the purpose of biomarker measurement a study in a case-cohort design was established (24, 25). In this study type, a random subcohort of the total baseline cohort is drawn as a reference group. If a subset member develops a disease of interest during follow-up, this person is also treated as a case and contributes to the exposure distribution among the cases (24). For the current analyses, a subcohort comprising 851 subjects free of MI and stroke at baseline was randomly drawn from the EPIC-Potsdam cohort (n = 26 761). Five incident MIs occurred among these subcohort members. For the present analyses we excluded 8 cases and 36 noncases without sufficient plasma or DNA samples, leaving a final case-cohort sample of 148 cases and a subcohort of 815 subjects, including 810 noncases and 5 cases.

Baseline examinations

A total of 30 mL venous blood was collected at baseline from each study participant during examinations for the cohort study at the Potsdam center. The plasma was separated from the blood cells within 2 h, and all samples were frozen at −80 °C until the time of analysis.

Standardized anthropometric measurements were performed by trained personnel (26). The body mass index (BMI; in kg/m²) was calculated. Lifestyle characteristics, including regular physical exercise and smoking history, were documented by trained interviewers during a computer-guided interview. Smoking status was expressed as “current smoker,” “nonsmoker,” and “former smoker.” Physical exercise was defined as the mean time spent on sporting activities during the summer and winter seasons (in h/wk). For the assessment of their medical history, the participants answered questions about past diseases and regular use of medication.

With the use of oscillometric devices (BOSO-Oscillomat; Bosch & Sohn, Jungingen, Germany), presence of hypertension was defined on the basis of the mean values of the second and third blood pressure measurements during baseline examinations as systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg or from the subjects’ reports, disease-specific medication, or verification from medical records. Definition of diabetes was based on subjects’ reports or disease-specific medication or verification from medical records.

At baseline, the dietary habits during the preceding year were assessed by a validated self-administered food-frequency questionnaire (27, 28). The food-frequency questionnaire included questions on frequency and portion size of 146 individual food items and on regular use of vitamin supplements (during the previous year); it was described in detail elsewhere (29).

Definition of MI

Every 2–3 y, the participants were asked to complete a mailed follow-up questionnaire that included sections about self-reported MI. Potential incident MIs were verified from medical records or death certificates, applying the criteria based on the WHO-MONICA (MONItoring CArdiovascular disease) study (Internet: http://www.ktl.fi/publications/monica/manual/part4/iv-1.htm). Thus, cases were defined as incident MIs, according to codes I21.0–I21.9 from the 10th revision of the International Classification of Diseases, occurring between the baseline examination and 30 April 2004.

Blood measurements

Blood samples of selected study subjects were dispatched to the Institute of Clinical Chemistry on dry ice to be analyzed for PLP, folate, and creatinine. Plasma folate was measured with the use of a commercial test assay (Roche Diagnostics, Mannheim, Germany). PLP was measured by HPLC with fluorescence detection with the use of a commercial test kit (Immundiagnostik GmbH, Bensheim, Germany). The normal range of this assay is >18.2 nmol/L. Creatinine was measured enzymatically on a random access autoanalyzer (Roche Diagnostics). Plasma homocysteine was measured by HPLC with fluorescence detection (30). Blood lipids and hsCRP were measured at the German Institute of Human Nutrition Potsdam-Rehbrücke. Total and HDL cholesterol and hsCRP were measured with the use of standard methods with reagents from Horiba ABX (Shefford,
United Kingdom). The intraassay CVs were 0.9% (total cholesterol), 1.2% (HDL cholesterol), and 2.6% (hsCRP), and the interassay CVs were 4.7%, 5.2%, and 7.9%, respectively.

**Statistics**

The statistical analysis was performed with the use of SAS software package, release 9.1 (SAS Institute, Cary, NC). All tests were 2-sided, \( P < 0.05 \) as the significance threshold. Associations of PLP with the plasma concentrations of hsCRP, HDL cholesterol, creatinine, folate, and cobalamin and also with dietary variables such as intakes of vitamin B-6 and folate were studied by calculating Spearman \( \rho \) coefficients. The effect of smoking on PLP was tested with the use of the general linear model with 3 smoking categories. Cox proportional hazard regression analysis was used for the examination of the relation between PLP and risk of MI. Age was the underlying time variable in the counting process, with entry defined as the subjects’ age at the time of recruitment and exit defined as age at the diagnosis of MI or censoring. As suggested by Prentice (24), the Cox models were modified to account for the case-cohort design. With the Prentice method, participants within the subcohort are given a weight of 1 at all times, whereas cases outside the subcohort have a weight of 1 at time of event and have weight of 0 at all other times (24). Hazard rate ratios were calculated for quintiles of PLP. Quintiles of PLP plasma concentrations are based on distribution of the subcohort. The lowest quintile was used as the reference category. The association of PLP with risk of MI was first calculated after adjustment for age and for sex. As a control for the known association of hsCRP on PLP, hsCRP was first introduced into the age- and sex-adjusted model. Because smoking also showed a significant association with PLP, a model containing age, sex, and smoking in 3 categories (never smoked, former smoker, current smoker) was constructed. In addition, a model of age, sex, and the ratio of total cholesterol to HDL cholesterol (total cholesterol:HDL cholesterol) was constructed. The effect of multiple risk factors on the association of PLP with risk of MI was then tested in a model consisting of age and sex, hsCRP, smoking, and total cholesterol:HDL cholesterol. To this model, other known risk factors for ischemic heart disease were then added, including calculated alcohol intake, regular exercise (<2 h/wk, \( \geq 2 \) h/wk), educational level (university degree, no university degree), and in addition BMI, hypertension, and diabetes mellitus. Tests for trends across increasing quintiles of PLP concentration were conducted by using the quintile number as a continuous variable in Cox proportional hazard models.

To evaluate putative interactions between PLP and important confounders, multiplicative interaction terms of PLP and sex, PLP and smoking, and PLP and hsCRP were included and tested in the fully adjusted Cox regression model. Furthermore, we investigated whether the effect of PLP on incidence of MI was changing over time, applying the test for proportionality assumption of the Cox model. The proportionality assumption was not rejected in our analyses, suggesting that the influence of PLP on incidence of MI did not change over time.

**RESULTS**

**Subject characteristics**

The current analysis is based on a nested case-cohort study consisting of 815 subjects in a subcohort of the EPIC Potsdam study and 148 subjects who had an MI during a mean follow-up of 6.0 ± 1.5 y. One hundred eleven cases were nonfatal and the remaining 37 were fatal.

Selected baseline characteristics according to PLP quintiles are presented in Table 1. Subjects with higher PLP concentrations were less likely to be men, to be current smokers, or to have a history of hypertension or diabetes at baseline than were subjects with low PLP concentrations. The plasma PLP at baseline correlated significantly with vitamin B-6 intake (\( r = 0.15, n = 958, P < 0.001 \)), hsCRP (\( r = -0.25, n = 958, P < 0.001 \)), homocysteine (\( r = -0.11, n = 958, P < 0.001 \)), both folate intake (\( r = 0.17, n = 958, P < 0.001 \)) and plasma folate (\( r = 0.31, n = 958, P < 0.001 \)) and HDL cholesterol (\( r = 0.10, n = 958, P < 0.01 \)). The association between PLP and hsCRP remained unchanged when 4 subjects with CRP > 20 mg/L (indicating acute inflammation) were excluded. Smoking exhibited a significant effect on plasma PLP, with lower concentrations in current smokers compared with former smokers or never smokers (Table 2).

**PLP and risk of MI**

In the Cox regression model adjusted for age and sex, higher quintiles of plasma PLP were significantly associated with a lower risk of MI (Table 3). The relative risk for subjects in the fifth quintile for plasma PLP was 0.50 (95% CI: 0.29, 0.83), compared with subjects with plasma PLP in the lowest quintile. This association was substantially weakened after adjustment for either hsCRP or smoking or for total cholesterol:HDL cholesterol. When the model was adjusted for the combination of these risk factors, the association of PLP and risk of MI was completely abolished.

Further adjustment for other factors known to be associated to coronary risk, such as alcohol intake, physical activity, educational level, hypertension, diabetes, or BMI did not change this result substantially (Table 3). Further adjustment for homocysteine did not change this result substantially (data not shown). When the Cox analysis was performed for PLP tertiles or quartiles, the associations between PLP and risk of MI became weaker. In fact, the association between tertiles or quartiles of PLP and risk of MI disappeared already after adjustment for hsCRP alone (data not shown). We did not observe any statistically significant interaction between PLP and sex, PLP and smoking, or PLP and hsCRP (data not shown).

**PLP compared with other risk factors**

In the fully adjusted model, established risk factors such as total cholesterol:HDL cholesterol, hypertension at baseline, smoking, and hsCRP still predicted MI. The hazard ratios were 1.66 (95% CI: 1.42, 1.96) for one unit increase of the total cholesterol:HDL cholesterol, 2.02 (95% CI: 1.31, 3.11) for hypertension at baseline, 3.03 (95% CI: 2.03, 4.53) for smoking, and 1.08 (95% CI: 1.01, 1.15) for one unit increase of hsCRP.

**DISCUSSION**

The present work is the first European prospective study on the association between plasma PLP and cardiovascular disease risk, after the publication of numerous retrospective studies and 2 American studies with nested case-control design (12, 13). The principal finding of the present nested case-cohort study is that the plasma PLP was not associated with future MI in a cohort of
German middle-aged men and women healthy at the time of entry into the study and after adjustment for known cardiovascular disease risk factors. This disagrees with the findings reported by other groups in studies with retrospective design (5, 8, 10) and with studies that also followed a nested case-control design (13), but it is in accord with earlier, retrospective studies (6, 7, 11), with studies that also followed a nested case-control design (13), and with one of the prospective studies (12).

The main reason to doubt the existence of a causal association between PLP and CVD was the increasing evidence of an effect of the inflammatory response on plasma PLP. This was first observed in elderly British subjects (15) and in British children (31), then in the Framingham cohort (14), and in persons with type 2 diabetes with nephropathy (18), but not in a random middle-aged subsample of the ARIC study (32). The association persisted in the studies even after the exclusion of subjects with acute inflammatory reactions. It seems that this result is independent of the manner of PLP quantification: by HPLC or by the radioenzymatic tyrosine decarboxylase method.

With the exception of Friso et al (10), most previous studies on PLP as a risk factor for CVD made no adjustment for the acute-phase response. However, higher CRP concentrations are usually observed in the cases included both in retrospective and prospective studies (10, 11, 19, 20). This evidently may influence the PLP concentration and may be able to imitate a strong association between PLP and CVD. Although we observed a strong inverse association between plasma PLP and hsCRP, adjustment for hsCRP alone did not abolish the association between PLP and risk of MI, a finding in line with Friso et al (10). In most studies, more patients with MI than control subjects usually have a history of smoking or are current smokers. We observed a strong effect of smoking on plasma PLP, although the association between PLP and future risk of MI was not abolished by introducing smoking alone into the model. Lower plasma PLP concentrations in smokers were reported earlier (33), but the consequences on the association between plasma PLP and risk of MI have not been systematically studied in detail. However, when hsCRP and smoking were introduced into the Cox regression model, the association of plasma PLP and MI was abolished (data not shown).

TABLE 1
Baseline characteristics of study participants in the European Investigation into Cancer and Nutrition (EPIC) Potsdam case-cohort study (n = 958) according to quintile (Q) of plasma pyridoxal-5-phosphate (PLP)\(^1\)

<table>
<thead>
<tr>
<th>PLP</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
<th>Q5</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcohort (n)</td>
<td>162</td>
<td>164</td>
<td>165</td>
<td>163</td>
<td>161</td>
<td></td>
</tr>
<tr>
<td>Cases [n (%)]</td>
<td>42 (21)</td>
<td>33 (17)</td>
<td>19 (10)</td>
<td>29 (15)</td>
<td>25 (13.5)</td>
<td>0.05</td>
</tr>
<tr>
<td>PLP (nmol/L)(^2)</td>
<td>8.1–18.0</td>
<td>22.3–30.3</td>
<td>30.4–39.2</td>
<td>39.6–54.6</td>
<td>55.0–550</td>
<td></td>
</tr>
<tr>
<td>PLP (nmol/L)</td>
<td>16.2 ± 4.00</td>
<td>26.3 ± 2.4</td>
<td>35.2 ± 2.4</td>
<td>46.9 ± 4.4</td>
<td>96.7 ± 80.1</td>
<td></td>
</tr>
<tr>
<td>Vitamin B-6 intake (mg/d)</td>
<td>1.53 ± 0.43</td>
<td>1.62 ± 0.42</td>
<td>1.66 ± 0.53</td>
<td>1.72 ± 0.47</td>
<td>1.72 ± 0.49</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

General characteristics
- Age (y) | 51.3 ± 9.3 | 50.1 ± 8.3 | 51.1 ± 7.8 | 50.7 ± 8.8 | 51.4 ± 8.7 | NS |
- Women [n (%)] | 124 (61) | 119 (61) | 107 (58) | 88 (46) | 87 (47) | 0.01 |
- University degree [n (%)] | 68 (34) | 74 (38) | 64 (35) | 74 (39) | 90 (49) | 0.09 |

Laboratory variables
- Folate (nmol/L) | 19.2 ± 6.6 | 21.5 ± 16.5 | 22.0 ± 8.4 | 24.0 ± 10.0 | 28.5 ± 14.7 | < 0.01 |
- Total-HDL cholesterol | 4.19 ± 1.23 | 4.06 ± 1.06 | 4.03 ± 1.02 | 4.19 ± 1.11 | 3.97 ± 0.99 | NS |
- hsCRP (mg/L) | 2.28 ± 3.02 | 1.97 ± 2.96 | 1.29 ± 0.80 | 0.97 ± 1.34 | 1.08 ± 2.24 | < 0.01 |
- hsCRP (mg/L)\(^4\) | 1.2 | 0.9 | 0.55 | 0.5 | 0.4 |     |
- Homocysteine (µmol/L) | 10.1 ± 4.5 | 8.8 ± 2.8 | 9.0 ± 4.1 | 8.8 ± 3.2 | 8.8 ± 3.3 | < 0.01 |

Baseline diseases and lifestyle factors
- Hypertension [n (%)] | 107 (53) | 100 (51) | 97 (53) | 84 (44) | 84 (46) | 0.05 |
- Diabetes [n (%)] | 13 (6) | 7 (4) | 6 (3) | 2 (1) | 3 (2) | < 0.01 |
- BMI (kg/m\(^2\)) | 26.1 ± 3.7 | 26.6 ± 4.0 | 26.0 ± 3.5 | 25.9 ± 3.2 | 25.6 ± 3.5 | NS |
- Smokers [n (%)]\(^5\) | 97 (47) | 55 (28) | 52 (27) | 61 (32) | 40 (22) | 0.05 |
- Alcohol intake (g/d) | 9 ± 11 | 12 ± 15 | 13 ± 14 | 17 ± 18 | 19 ± 19 | < 0.01 |
- Alcohol (g/d)\(^6\) | 5 | 7 | 9 | 12 | 12 |     |
- Regular exercise [n (%)]\(^6\) | 26 (13) | 35 (18) | 31 (17) | 38 (20) | 36 (19) | NS |
- Vitamin supplement use [n (%)] | 25 (12) | 24 (12) | 25 (14) | 34 (18) | 59 (32) | < 0.01 |

\(^1\) Quintiles are based on the distribution of PLP in control subjects (subcohort). hsCRP, highly sensitive C-reactive protein.
\(^2\) Values are ranges.
\(^3\) x ± SD (all such values).
\(^4\) Medians are used because of the highly skewed distributions.
\(^5\) Includes current and former smokers.
\(^6\) Defined as >2 h/wk.

TABLE 2
Association between smoking and plasma pyridoxal-5-phosphate (PLP)\(^1\)

<table>
<thead>
<tr>
<th>Smoking category</th>
<th>PLP concentrations (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Never smokers (n = 655)</td>
<td>46.5 (43.3, 50.2)</td>
</tr>
<tr>
<td>Former smokers (n = 68)</td>
<td>37.2 (26.3, 47.7)</td>
</tr>
<tr>
<td>Current smokers (n = 235)</td>
<td>36.4 (30.7, 42.1)</td>
</tr>
</tbody>
</table>

\(^1\) All values are x; 95% CI in parentheses. An association was observed between smoking category and PLP concentration, P < 0.001.
We also observed an association between HDL cholesterol and plasma PLP concentrations, especially in men. To our knowledge, no studies in the general population reported this association earlier. This is therefore an interesting observation that deserves future investigations.

The idea that high plasma PLP concentrations may be cardioprotective is indeed supported by some evidence derived from biochemical studies. PLP is a coenzyme of numerous biological pathways in the carbohydrate, amino acid, protein, and lipid metabolism. This offers a great number of mechanisms that may contribute to an antiatherogenic action of PLP. Among these, PLP is a coenzyme of d6-desaturase, involved in the metabolism of polyunsaturated fatty acids (34, 35), and a coenzyme of cystathionine-β-synthase involved in methionine, homocysteine, and cysteine metabolism (36). Furthermore, it can occupy glycoprotein IIb or IIa, a major receptor on platelets responsible for platelet aggregation (3), and it was shown that PLP can inhibit proliferation of endothelial cells (37).

Epidemiologic studies on vitamin B-6 intake, however, are also inconsistent about whether a higher intake of vitamin B-6 is antiatherogenic. In the Nurses’ Health Study, a higher intake of vitamin B-6 was associated with a reduced risk of MI (38). However, the effect was mostly due to the use of supplements and probably due to the oversampling of women in the cohort and to the relatively short time of follow-up. However, we believe that the conclusions drawn are valid although the number of cases is relatively low. Established risk factors such as total cholesterol: HDL cholesterol, hypertension at baseline, smoking, or hsCRP remained predictive for MI even in the fully adjusted model.

In conclusion, our results suggest that the association of plasma PLP with cardiovascular disease risk is mainly explained by other risk factors that lead to low plasma PLP concentrations. In particular, high hsCRP (a measure of inflammation) and smoking had an association with low plasma PLP concentrations.
epidemiologic studies other means of vitamin B-6 status (both measurements of plasma PLP and whole-blood PLP, or plasma or urinary pyridoxic acid), and major confounders should be used if possible to clarify the association between vitamin B-6 and CVD.

We thank Elke Hinze for the laboratory measurements, Ellen Kohlsdorf and Wolfgang Bernigau for data management, Wolfgang Fleischhauer for ascertaining the MI cases, and Kurt Hoffmann for assistance with the statistics. The author’s responsibilities were as follows—JD and CW: wrote the first draft of the manuscript, conducted the statistical analysis, and contributed equally; JD, SW, CL, MM, and JS: performed the laboratory measurements; HF: planned and conducted the EPIC-Potsdam Study; KK-G: contributed to the study design, acquisition, and interpretation of data in the EPIC-Potsdam Study. All authors critically revised the manuscript for important intellectual content. None of the authors had a conflict of interest in relation to this study.

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Dietary patterns and cardiovascular mortality in the Melbourne Collaborative Cohort Study1–3

Linton R Harriss, Dallas R English, John Powles, Graham G Giles, Andrew M Tonkin, Allison M Hodge, Laima Brazionis, and Kerin O’Dea

ABSTRACT

Background: Despite increased cardiovascular disease risk factors, migrants to Australia from Mediterranean countries have lower mortality than do native-born Australians. Dietary patterns may contribute to this.

Objective: The objective was to investigate the relation between dietary patterns and mortality from cardiovascular (CVD) and ischemic heart disease (IHD) in an ethnically diverse population.

Design: This was a prospective cohort study (mean follow-up: 10.4 y) of 40 653 volunteers (23 980 women) aged 40–69 y in the Melbourne Collaborative Cohort Study (1990–1994); 24% of the subjects were Mediterranean born.

Results: Four dietary factors were identified from a food-frequency questionnaire with the use of principal components analysis. They explained 69% of intake variance and reflected frequent intakes of Mediterranean foods, vegetables, meat, and fresh fruit. The Mediterranean factor was inversely associated with CVD and IHD mortality in models adjusting for diabetes, waist-to-hip ratio, body mass index, and hypertension. For IHD, the hazard ratio (HR) for the highest compared with the lowest quartile of consumption was 0.59 (95% CI: 0.39, 0.89; P for trend = 0.03). Associations persisted in analyses excluding people with prior CVD (HR: 0.51; 95% CI: 0.30, 0.88; P for trend = 0.03). Vegetable and fresh fruit factors were inversely associated with CVD mortality but only among those without prior CVD. HRs (highest compared with lowest quartile) were 0.66 (95% CI: 0.48, 0.92; P for trend = 0.02) for vegetables and 0.69 (95% CI: 0.52, 0.93; P for trend = 0.04) for fresh fruit. The meat factor was not associated with CVD or IHD mortality.

Conclusion: Our findings suggest that frequent consumption of traditional Mediterranean foods is associated with reduced cardiovascular mortality after controlling for important risk factors and country of birth. Am J Clin Nutr 2007;86:221–9.

KEY WORDS Diet, factor analysis, statistical analysis, food habits, cardiovascular disease, coronary heart disease, mortality, prospective studies, Mediterranean diet

INTRODUCTION

Despite large declines in age-adjusted rates, cardiovascular disease (CVD) remains the single largest cause of death and disability in Australia for both men and women (1). Continuing declines are possible and can be achieved through modification of individual behavior. For example, cardiovascular risk factors, including diabetes, dyslipidemia, hypertension, and overweight, can be influenced by dietary habits. Although migrants to Australia from Mediterranean countries have high prevalence of these risk factors, they have lower CVD mortality than native-born Australians (2). It is not clearly understood why this paradox exists. A possible reason for this observation is the traditional Mediterranean diet which is associated with higher intakes of plant foods and fish, moderate intake of wine, and lower intake of animal products. As such, it is a rich source of bioactive phytochemicals, monounsaturated fats, n–3 fatty acids, and fiber and is relatively low in saturated fats. In combination, these characteristics are believed to have antioxidant, anti-inflammatory, and antithrombotic properties that may counterbalance adverse effects of CVD risk factors (3).

Epidemiologic studies addressing diet have focused predominantly on identifying individual dietary constituents that might predict disease (4). However, the exact mechanisms responsible for the effects of these individual nutrients remain poorly understood (5). Nutrients are not consumed in isolation, and bioavailability is often determined by the interaction and combination of foods consumed at any one time (6). The effects of single foods or nutrients may be too small to measure, but effects of combinations may be measurable in epidemiologic studies. For these reasons, research into dietary patterns provides a practical and complementary approach to study dietary effects on disease outcomes. Two reviews show the growing interest in such studies (7, 8). Since 1990, 5 studies have investigated the association

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2 Supported by VicHealth and The Cancer Council Victoria for cohort recruitment, by grants from the National Health and Medical Research Council (284476, 209057, 124317, and 251533) and VicHealth (supplement grant 2003-0759), and by The Cancer Council Victoria and Monash University for further infrastructure support.

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between dietary patterns and total CVD or ischemic heart disease (IHD) mortality (9–13). In general, those studies support the contention that healthier dietary patterns (characterized by fruit, vegetables, and whole grains) are cardioprotective, whereas those deemed less healthy (characterized by meat products, high-fat and processed foods) are associated with elevated cardiovascular risk. The predominant feature of those studies, however, is their tendency to investigate only 2 dietary factors with the use of a relatively small number of food items or predefined food groups. Further, with the exception of one other study (13), cohort participants tend to be culturally similar, which may result in homogeneity of dietary intake. These attributes influence the composition of dietary factors and may therefore limit interpretation of results (14).

In this study, we use data from a food-frequency questionnaire (FFQ) containing 121 food items collected from an Australian cohort with a high proportion of middle-aged Mediterranean-born migrants. We used factor analysis to identify 4 main dietary patterns and prospectively investigated the relation between these dietary factors and total CVD and IHD mortality.

SUBJECTS AND METHODS

Study population

The Melbourne Collaborative Cohort Study is a prospective cohort of 41 528 participants (24 479 women) aged 40–69 y at baseline. Full details of the design, recruitment, and study procedures were published previously (15). Recruitment occurred from 1990 to 1994 by the electoral roll, ethnic radio, clubs, and churches. Twenty-four percent of subjects were southern European migrants, deliberately oversampled to extend the range of dietary and lifestyle exposures and genetic variation. The Cancer Council Victoria Human Research Ethics Committee approved the study, and subjects gave their written informed consent to participate.

At baseline, face-to-face interviews were conducted in the language of choice, and questionnaires addressing lifestyle exposure, including dietary intake, were completed. Subjects missing data for dietary intake and any of the final covariates modeled were excluded from analyses. Those who had daily energy intake divided by the number of people in the household to give 0.01 indicated no intake or less than once per month. Intake of alcohol as beer, wine, and spirits. Garlic intake was measured on a transformed scale in which a score of 1 indicated daily use, and a score of 6 or more times per day. This left 40 653 subjects for final analysis (23 980 women, 16 673 men).

Assessment of dietary intake

Dietary information was collected with the use of a self-administered, 121-item FFQ designed to assess the diverse diet in the cohort, over the preceding year (16). Nine possible frequency responses were available for each food item, ranging from “never or less than once per month” to “6 or more times per day.” This information was then used to calculate daily equivalent frequency. Vitamin supplements were not included as food items. Repeatability analyses for the FFQ have shown fair-to-moderate agreement for food items when completed 12 mo apart (17). A recent study that used the FFQ data found moderate-to-strong correlations between dietary intake of monounsaturated and polyunsaturated fatty acids and plasma phospholipid fatty acids (18). A similar study found that estimated dietary antioxidants (α-carotene, β-carotene, β-cryptoxanthin, lutein or zeaxanthin, and lycopene) were useful when predicting plasma antioxidant concentrations (JA Simpson, K O’Dea, DR English, et al, unpublished observations, 2006).

Baseline demographic and risk factors

Potential confounders determined at baseline were used in multivariate regression analyses. These confounders included country of birth (Australia, United Kingdom, Italy, and Greece), smoking (never, current, and former), total daily energy intake (continuous variable as kcal), physical activity (4 categories that used a score combining frequency and intensity of exercise in the past 6 mo), highest education level obtained (primary, some secondary, secondary school, and tertiary qualification), history of CVD (angina, myocardial infarction, or stroke), family history of CVD (mother, father, sister, or brother), history of diabetes, history of hypertension, social isolation (men living alone or who have no social activities, men and women who have no friends they could visit without an invitation), waist-to-hip ratio (WHR), body mass index (BMI; in kg/m²), and sex.

Ascertainment of deaths

Deaths were included in the analysis if they occurred between the recruitment date and 31 December 2003. By this date, 51 (0.13%) participants (29 Australian, 10 English, 2 Italian, and 10 Greek) had moved from Australia. Mortality definitions were based on primary cause of death with the use of the codes for CVD (390–459 and I00–I99) and IHD (410–414 and I20–I25), respectively, from the 9th and 10th revisions of the International Classification of Diseases (19, 20). The Victorian Cancer Registry assigned codes for underlying cause of death for deaths that occurred in 1991 and 1992. All other deaths were coded by the Australian Bureau of Statistics.

Statistical analysis

Analyses were performed with the use of STATA 8.2 (StatCorp, College Station, TX). Factor analysis was used to determine the main dietary factors from the 121 food items. Six additional food items were calculated from separate questions. These items included intake of garlic, olive oil, vegetable oil, and alcohol as beer, wine, and spirits. Garlic intake was measured on a transformed scale in which a score of 1 indicated daily use, and 0.01 indicated no intake or less than once per month. Intake of olive and vegetable oils was calculated from total household intake divided by the number of people in the household to give mL/wk. Consumption of alcoholic beverages was measured as grams of alcohol per day.

Dietary factors were calculated by using data from all 40 653 eligible subjects. Orthogonal (varimax) rotation was then used to generate uncorrelated factors for greater interpretability. We determined the number of main dietary factors by choosing those accounting for the greatest variation of dietary intake and by graphical assessment of the Scree plot. Four factors with eigenvalues > 2 were retained. These accounted for 69% of total intake variance. Factor loadings with absolute values ≥ 0.20 were used to interpret the factors. Subjects received a factor score for each dietary factor, and these scores were divided into quartile groups for analyses.

Cox proportional hazards models were constructed with age as the time axis (21). Follow-up began at baseline and ended at the time of death, date left Australia, or 31 December 2003, whichever came first. Diabetes, WHR, BMI, and hypertension may be
mediators of any relation between diet and CVD. We therefore performed 2 sets of analyses, first excluding and then including these covariates. Adjusted hazard ratios (HRs) were calculated for each dietary factor with the lowest quartile as the reference group. Confidence limits were set at the 95% level and two-sided \( P \) values are presented. A \( P \) value < 0.05 was regarded as significant. The proportional hazards assumption was tested for all variables in the model. Violation of the test occurred for variables representing history of CVD, family history of CVD, and sex. Final analyses were therefore stratified by these covariates. Tests of linear trend were performed across dietary factor categories by constructing a continuous variable assigning median values from each category. Tests for interaction were performed for country of birth (as a dichotomous variable using Australia and United Kingdom compared with Italy and Greece), sex, diabetes, and history of CVD.

Several sensitivity analyses were completed to determine the robustness of our results. First, the statistical analyses were repeated after removing those with a baseline history of CVD (\( n = 2449 \)). Second, we performed factor and regression analyses for non–Mediterranean and Mediterranean-born subjects separately. Four factors were created by using each of the non-Mediterranean and Mediterranean subcohorts. All 4 factors from the non-Mediterranean subjects, and 3 factors from the Mediterranean subjects were similar and highly correlated with factors created by using all subjects. The fourth factor from the Mediterranean-born subjects was not correlated with any other factor. This factor was characterized by frequent intake of items, including salami, hard cheeses, sweet biscuits, ham, bacon, pasta, white bread, sausage or frankfurters, vegetable or chicken dishes, chocolate, corn or potato crisps, ice cream, soft drinks, tinned fish, peanut butter or peanuts, margarine, butter, and other confectionery. Adjusted HRs (CVD and IHD) for the highest compared with the lowest quartile of consumption for each of the 3 similar factors were consistent for each subgroup compared with those that used all subjects. None of the results reached statistical significance, and CIs were considerably wider, especially for the Mediterranean-born group. For these reasons, we opted to use data from all subjects for the main analyses.

RESULTS

Dietary factors

Four main factors emerged with the use of the factor analysis procedure. Food items that had rotated factor loadings with absolute values of 0.2 or greater are given in Table 1. The first factor (Mediterranean) was characterized by frequent intake of items, including garlic, cucumber, olive oil, salad greens, capsicum, cooked dried legumes, legume soups, feta and ricotta cheeses, olives, steamed fish, and boiled chicken. This factor was also negatively associated with consumption of tea, margarine, sweet biscuits, and cake. Factor 2 (vegetables) was characterized by frequent intake of cauliflower, broccoli, carrot, cabbage or Brussels sprouts, pumpkin, green beans or peas, leafy greens, celery or fennel, potato cooked without fat, beetroot, zucchini or squash or eggplant, coleslaw, salad greens, cucumber, and capsicum. Factor 3 (meat) was associated with frequent intake of beef rissoles, roast beef or veal, fried potato, beef or veal schnitzel, savory pastries, mixed dishes with lamb, fried eggs, beef

<table>
<thead>
<tr>
<th>Food item</th>
<th>Factor 1 (Mediterranean foods)</th>
<th>Factor 2 (vegetables)</th>
<th>Factor 3 (meat)</th>
<th>Factor 4 (fresh fruit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garlic</td>
<td>0.44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cucumber</td>
<td>0.41</td>
<td>0.32</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Olive oil</td>
<td>0.41</td>
<td>0.32</td>
<td>0.21</td>
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</tr>
<tr>
<td>Salad greens</td>
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<td>0.32</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>Capsicum</td>
<td>0.35</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cooked dried legumes</td>
<td>0.32</td>
<td>0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Legume soups</td>
<td>0.31</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Tomato</td>
<td>0.29</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pasta dish</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olives</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>Celery or fennel</td>
<td>0.28</td>
<td>0.43</td>
<td></td>
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<tr>
<td>Feta cheese</td>
<td>0.28</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Beef or veal schnitzel</td>
<td>0.27</td>
<td>0.34</td>
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<tr>
<td>Ricotta cheese</td>
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<td></td>
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<td>Steamed fish</td>
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<td>0.21</td>
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<td>Onion or leek</td>
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<td>Boiled or steamed chicken</td>
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<tr>
<td>Leafy greens</td>
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<td>0.46</td>
<td></td>
<td></td>
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<tr>
<td>Game</td>
<td>0.23</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Zucchini, eg, parmesan</td>
<td>0.23</td>
<td></td>
<td>0.23</td>
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</tr>
<tr>
<td>Hard cheese, eg, parmesan</td>
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<td>0.23</td>
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<td>Watermelon</td>
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<tr>
<td>Cauliflower</td>
<td>0.60</td>
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<td></td>
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<tr>
<td>Broccoli</td>
<td>0.59</td>
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<tr>
<td>Carrot</td>
<td>0.58</td>
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<td></td>
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<tr>
<td>Cabbage or Brussel sprouts</td>
<td>0.57</td>
<td></td>
<td>0.56</td>
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</tr>
<tr>
<td>Pumpkin</td>
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<td></td>
</tr>
<tr>
<td>Green beans or peas</td>
<td>0.48</td>
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<td>0.48</td>
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</tr>
<tr>
<td>Beetroot</td>
<td>0.42</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Zucchini or squash or eggplant</td>
<td>0.34</td>
<td></td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Coleslaw</td>
<td>0.33</td>
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</tr>
<tr>
<td>Whole-meal bread</td>
<td>0.26</td>
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<td>0.26</td>
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</tr>
<tr>
<td>Banana</td>
<td>0.26</td>
<td>0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mushroom</td>
<td>0.24</td>
<td></td>
<td></td>
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<tr>
<td>Yogurt</td>
<td>0.24</td>
<td></td>
<td></td>
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<tr>
<td>Vegetable dish</td>
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</tr>
<tr>
<td>Sweet corn</td>
<td>0.23</td>
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</tr>
<tr>
<td>Pineapple</td>
<td>0.22</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit salad</td>
<td>0.22</td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken dish</td>
<td>0.21</td>
<td>0.21</td>
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<td></td>
</tr>
<tr>
<td>Beef rissoles</td>
<td>0.40</td>
<td>0.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef or veal roast</td>
<td>0.39</td>
<td></td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Potato cooked in fat</td>
<td>0.36</td>
<td></td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Savory pastries</td>
<td>0.34</td>
<td></td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Mixed dishes with lamb</td>
<td>0.33</td>
<td></td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Fried eggs</td>
<td>0.33</td>
<td>0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef steaks</td>
<td>0.33</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Fried fish</td>
<td>0.31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacon</td>
<td>0.31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pork roast</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White bread</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef dish</td>
<td>0.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roast or fried chicken</td>
<td>0.27</td>
<td></td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Egg dish</td>
<td>0.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comed beef</td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fried rice</td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apricots</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peach or nectarine</td>
<td>0.66</td>
<td></td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>Plums</td>
<td>0.64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cantaloupe or honeydew</td>
<td>0.55</td>
<td></td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Grapes</td>
<td>0.55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pears</td>
<td>0.53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strawberries</td>
<td>0.46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orange or mandarin</td>
<td>0.43</td>
<td></td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Figs</td>
<td>0.42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apples</td>
<td>0.40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cream or sour cream</td>
<td>–0.20</td>
<td></td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>Lamb or chops</td>
<td>–0.23</td>
<td>0.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other cereal</td>
<td>–0.21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ice cream</td>
<td>–0.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chocolate</td>
<td>–0.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sausage or frankfurter</td>
<td>–0.25</td>
<td>0.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jam or honey</td>
<td>–0.26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potato cooked without fat</td>
<td>–0.26</td>
<td>0.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pudding</td>
<td>–0.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cakes or sweet pastries</td>
<td>–0.31</td>
<td></td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Sweet biscuits</td>
<td>–0.31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Margarine</td>
<td>–0.36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tea</td>
<td>–0.37</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Variance of intake explained (%) | 28.2 | 17.6 | 12.4 | 10.4 |

1 Dietary factors were determined by factor analysis.
2 Factor loadings with absolute values < 0.20 were omitted for simplicity.
Country of birth explained 36% of the variation in the Mediterranean factor, with 69% of those in the highest quartile born in Italy or Greece (Table 2). Education explained 20% of the variation in the Mediterranean factor, with 51% of those in the highest quartile having completed primary school only. This is because the Mediterranean-born subjects had lower levels of formal education than did Australian-born subjects. Country of birth explained 11% of variation in the vegetable factor, with 90% of those in the top 2 quartiles born in Australia or the United Kingdom. Thirty-three percent of the variation in daily energy intake was explained by the meat factor, with those in the highest quartile having the greatest mean total daily energy intake.

Mortality

After a mean follow-up of 10.4 y, 697 CVD deaths, including 407 from IHD, had occurred (Table 3). In model 1 (without adjustment for diabetes, WHR, BMI, and hypertension) none of the factors showed significant associations with CVD or IHD mortality. After further adjustment for other covariates (model 2), the Mediterranean factor was inversely associated with both CVD and IHD death. The fresh fruit factor was weakly inversely associated with CVD death, although the linear trend was not significant.

Tests of interaction between dietary factors and mortality for country of birth, sex, and history of CVD were not significant. However, history of diabetes modified the relation between IHD mortality and the Mediterranean and fresh fruit factors (P for interaction = 0.03 and 0.04, respectively). A borderline interaction was observed for history of diabetes and the vegetable factor (P interaction = 0.06). IHD data were then reanalyzed to investigate the association between these 3 factors and persons with diabetes. Analyses for the Mediterranean and vegetable factors gave notably different results among persons with diabetes than did the remainder of the cohort without diabetes. Compared with quartile 1, HRs for the Mediterranean factor were 0.42 (95% CI: 0.18, 0.97) for quartile 2, 0.40 (95% CI: 0.17, 0.97) for quartile 3, and 0.21 (95% CI: 0.09, 0.47) for quartile 4. As a result of this finding, the overall association observed among persons without diabetes was slightly weakened for the Mediterranean factor when compared with the model controlling for the presence of diabetes (HR for the highest compared with the lowest quartile of intake: 0.71; 95% CI: 0.47, 1.08). For the vegetable factor, HRs for people with diabetes were 2.32 (95% CI: 1.02, 5.26) for quartile 2, 2.50 (95% CI: 1.07, 5.84) for quartile 3, and 1.74 (95% CI: 0.71, 4.27) for quartile 4 when compared with quartile 1. For the fresh fruit factor, no association for IHD was observed in either stratum of the diabetes variable.

Among participants without a baseline history of CVD (Table 4), 473 CVD deaths, including 242 from IHD, occurred since baseline. In model 1 (without diabetes, WHR, BMI, and hypertension) both the vegetable and fresh fruit factors were inversely associated with CVD mortality. After further adjustment for other covariates (model 2), these associations remained virtually identical, and the Mediterranean factor was inversely associated with both CVD and IHD death.

DISCUSSION

We identified 4 main dietary factors with the use of factor analysis, which appeared to represent intakes of Mediterranean foods, vegetables, meat, and fresh fruit. After adjusting for established risk factors, the Mediterranean factor was inversely associated with CVD and IHD mortality in models adjusting for CVD history, diabetes, WHR, BMI, and hypertension. The Mediterranean, vegetable, and fresh fruit factors were inversely associated with CVD mortality among those without previous history of CVD. Frequent intake of meat was not associated with CVD or IHD mortality. Among persons with diabetes, the Mediterranean factor was strongly associated with reduced IHD mortality risk, whereas those with high scores for vegetable intake had increased risk.

This is a large prospective study with an average of 10.4 y follow-up and minimal loss to follow-up. The ethnically diverse cohort ensured heterogeneity of dietary intake and, importantly, resulted in 4 distinct dietary factors: Mediterranean foods, vegetables, meat, and fresh fruit. Although baseline measurement of established cardiovascular risk factors was thorough, unknown potential confounders may also have influenced our findings. Reliability of memory is recognized as the core problem in dietary measurement (22); however, other factors may be involved such as interpretation of portion size, integrating intake over time to fit frequency responses, and interpreting the FFQ items in relation to the foods actually eaten. Sex, culture, health status, education, and questionnaire design may also have an effect. Consequently, the level of exposure misclassification likely to have been associated with reliance on a FFQ will have tended to bias point estimates toward the null. Furthermore, our FFQ was designed to measure dietary intake for the 12 mo before baseline, which may not have been representative of intake over the causative relevant period. In addition, repeatability analyses for the FFQ showed only fair-to-moderate agreement for food items when completed 12 mo apart (17). Finally, misclassification of cause of death could have affected the associations observed for dietary factors.

Aside from dietary measurement error, the subjective nature of factor analysis itself may influence the importance of dietary factors produced (14). Of particular importance are the dietary items included for analysis, the number of factors chosen to be extracted, and the value of the factor loading chosen to describe the factor. Furthermore, dietary factors derived from factor analysis depend on the population being studied, making direct comparisons between studies difficult (4). Most studies focusing on dietary patterns and CVD have used only 2 factors for analysis, “prudent” and “Western,” with the latter label apparently not encompassing components of the Mediterranean diet. These studies also regrouped their FFQ items before doing factor analysis, which requires the researcher to make some judgment about how individual food items go together. We allowed the data to be grouped without making any assumptions about how items were to be combined.

In Denmark, a study analyzing 2994 men and 2877 women separately (10) found that a prudent diet (characterized by frequent intakes of whole-meal bread, porridge, grains, and oats; pasta; rice; raw vegetables; boiled vegetables; fruit; juices; jam and honey; cakes and cookies; candy and chocolate; milk and yogurt; fish; low-fat margarine; and tea) was associated with reduced CVD death in women but not men. A Western diet factor

steaks, fried fish, and bacon. Factor 4 (fresh fruit) was characterized by frequent intake of apricots, peaches or nectarines, plums, cantaloupe or honeydew, grapes, pears, strawberries, oranges or mandarins, figs, apples, and pineapple.

After a mean follow-up of 10.4 y, 697 CVD deaths, including 407 from IHD, had occurred (Table 3). In model 1 (without adjustment for diabetes, WHR, BMI, and hypertension) none of the factors showed significant associations with CVD or IHD mortality. After further adjustment for other covariates (model 2), the Mediterranean factor was inversely associated with both CVD and IHD death. The fresh fruit factor was weakly inversely associated with CVD mortality. After further adjust-
Characteristics of 40,653 Australian men and women aged 40–69 y at baseline (1990–1994) who participated in the Melbourne Collaborative Cohort Study, according to dietary factor quartiles

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Factor 1 quartiles: Mediterranean foods</th>
<th>Factor 2 quartiles: vegetables</th>
<th>Factor 3 quartiles: meat</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Country of birth</strong></td>
<td>Australia 92 86 74 70 87 98 88 80</td>
<td>United Kingdom 63 61 51 53</td>
<td>Italy 27 32 48 30</td>
</tr>
<tr>
<td><strong>Education</strong></td>
<td>Highest 63 48 32 26</td>
<td>Completed high school 51 46 38 32</td>
<td>Tertiary school 39 31 24 20</td>
</tr>
<tr>
<td><strong>Family history of CVD</strong></td>
<td>None 65 62 55 49</td>
<td>Low 63 62 54 47</td>
<td>Medium 51 48 41 35</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>Women 75 72 69 66</td>
<td>Men 22 19 14 11</td>
<td></td>
</tr>
<tr>
<td><strong>History of diabetes</strong></td>
<td>None 0 0 0 0</td>
<td>Low 0 0 0 0</td>
<td>Medium 0 0 0 0</td>
</tr>
<tr>
<td><strong>History of hypertension</strong></td>
<td>None 0 0 0 0</td>
<td>Low 0 0 0 0</td>
<td>Medium 0 0 0 0</td>
</tr>
<tr>
<td><strong>Smoking status</strong></td>
<td>Never 66 65 62 60</td>
<td>Current 22 21 18 16</td>
<td>Former 12 13 16 18</td>
</tr>
<tr>
<td><strong>Social isolation</strong></td>
<td>None 25 24 23 22</td>
<td>Low 16 17 18 19</td>
<td>Medium 10 11 12 13</td>
</tr>
<tr>
<td><strong>Waist-to-hip ratio</strong></td>
<td>0.84 0.84 0.84 0.84</td>
<td>0.84 0.84 0.84 0.84</td>
<td>0.84 0.84 0.84 0.84</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>23.1 23.1 23.1 23.1</td>
<td>23.1 23.1 23.1 23.1</td>
<td>23.1 23.1 23.1 23.1</td>
</tr>
</tbody>
</table>

Dietary patterns were determined by factor analysis with the use of baseline food-frequency questionnaire data. CVD, cardiovascular disease.

Values are % of total number.

P for trend values are based on binary variable for country of birth (Australia and United Kingdom compared with Italy and Greece) and smoking (never or former compared with current).

Diary factors were determined by factor analysis with the use of baseline food-frequency questionnaire data. CVD, cardiovascular disease.

Values are % of total number.

P for trend values are based on binary variable for country of birth (Australia and United Kingdom compared with Italy and Greece) and smoking (never or former compared with current).
### TABLE 3
Hazard ratios (95% CIs) for the association between dietary factor quartiles (Qs) and death from all cardiovascular diseases (total CVD) and ischemic heart disease (IHD), during mean follow-up of 10.4 y, for 40 653 Australian men and women aged 40–69 y

<table>
<thead>
<tr>
<th>Dietary factor</th>
<th>Subjects</th>
<th>Person-years</th>
<th>Model 1</th>
<th>Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total CVD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mediterranean foods</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q1</td>
<td>214</td>
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1 Hazard ratios were calculated with the use of Cox proportional hazards regression.
2 Dietary factors were determined by factor analysis with the use of baseline food-frequency questionnaire data (1990–1994).
3 Hazard ratios were adjusted for country of birth, activity, daily energy intake, education, smoking, social isolation, and dietary factors, stratifying by sex, CVD history, and family history of CVD.
4 Model 1 with further adjustment for diabetes, waist-to-hip ratio, BMI, and hypertension.
TABLE 4
Hazard ratios (95% CIs) for the association between dietary factor quartiles (Qs) and death from all cardiovascular diseases (total CVD) and ischemic heart disease (IHD), among subjects without prior history of CVD, during a mean follow-up of 10.4 y for 38 204 Australian men and women aged 40–69 y.

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<th>Dietary factor</th>
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<th>Model 2</th>
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1 Hazard ratios were calculated with the use of Cox proportional hazards regression.
2 Dietary factors were determined by factor analysis.
3 Hazard ratios were adjusted for country of birth, activity, daily energy intake, education, smoking, social isolation, and dietary factors, stratifying by sex, CVD history, and family history of CVD.
4 Model 1 with further adjustment for diabetes, waist-to-hip ratio, BMI, and hypertension.
(characterized by frequent intakes of white bread, potatoes, juices, jam and honey, cakes and cookies, candy and chocolate, ice cream and soft drinks, milk and yogurt, eggs, meat, sausages, liver paste and cold meat, butter, lard, and margarine) was not associated with CVD mortality in either men or women. We chose not to do separate analyses for men and women because of power considerations and also because there was no evidence of effect modification by sex.

The Nurses’ Health Study (11, 23) used factor analysis to identify 2 dietary factors. The prudent pattern (characterized by frequent intake of vegetables, fruit, fish, poultry, and whole grains) was associated with reduced risk of IHD and stroke. The Western pattern (characterized by frequent intake of refined grains, red and processed meat, desserts, sweets, and full-fat dairy products) was associated with increased risk of IHD and stroke. The Health Professional’s Study of 44 875 US men produced 2 similar factors (12). Again, the prudent pattern was associated with reduced IHD risk, and the Western pattern was associated with increased risk.

Our Mediterranean factor, which was inversely associated with CVD and IHD mortality, was most similar to the prudent pattern described earlier. If these inverse associations indicate cause and effect, there are several candidate mechanistic hypotheses that could account for this. These include the role of antioxidants which are abundant in these foods. Phytochemicals such as bioflavonoids and carotenoids (a group of fat-soluble plant pigments such as α-carotene, β-carotene, and lycopene) and vitamins such as E and C were shown to be inversely associated with CVD (6). The null results from supplementation trials with antioxidant vitamins indicate that the health benefits of plant food intake cannot be attributed to individual micronutrients with antioxidant activity (24, 25). Many qualities of a diet rich in plant foods could contribute to the health benefits. For example, vegetable protein was shown to reduce serum cholesterol concentrations (26), and certain plant nutrients, including potassium, calcium, magnesium, and fiber, can reduce blood pressure (27–32). Folate, which is found in leafy green vegetables, citrus fruits, dried beans and peas, is important for lowering homocysteine concentrations which is a possible risk factor for IHD (33), although results from clinical trials testing folate have been null (34). Our Mediterranean factor was also characterized by the infrequent intake of certain foods as cream, sour cream, ice cream, chocolate, sausages, jams, honey, cake, and sweet biscuits. These foods are low in fiber and high in saturated fats, refined carbohydrates, and salt. Diets with these attributes were associated with increased cardiovascular risk (6). Therefore, the avoidance of these foods in the Mediterranean factor may partially explain the cardiovascular benefits observed in our study, especially among persons with diabetes.

We found no association between the frequent intake of meat and CVD or IHD mortality. This is similar to the Danish study (10) but unlike the 2 US studies (11, 12) in which the Western pattern was clearly associated with increased IHD mortality. If our results are correct, a possible explanation may be that cardioprotection is mediated primarily through plants. Diets high in meat products may therefore displace the consumption of more beneficial plant foods, thus reducing the cardioprotective potential of the overall diet (35).

We found that the associations were stronger for persons without a baseline history of CVD. Persons with previous disease are more likely to have modified their dietary habits as a consequence of illness and medical advice. For example, in our study, those with a history of CVD reported significantly lower intake of saturated fats than did the rest of the cohort, as well as higher intakes of fiber, polyunsaturated fats, and fish, and their reported diets might not be representative of those at the causative relevant time. It is also possible that other nondietary risk factors (not measured) operate after disease is established. However, if dietary risk factors for initial disease events are different from those for subsequent disease progression, previous disease would be an effect modifier, and this was not found in our study. However, tests for effect modification are often underpowered. We believe there is real plausibility to expect that history of CVD does modify the relation between diet and mortality in these analyses.

Our results suggest that the more frequent consumption of foods from the Mediterranean pattern may reduce CVD and IHD risks and may be most beneficial for persons with diabetes. Frequent intake of foods from the vegetable and fresh fruit patterns may reduce CVD risk for those without a prior history of CVD. In addition, frequent intake of meat does not increase risk after controlling for established risk factors. These findings are consistent with current recommendations to increase daily intakes of fruit and vegetables (6). Future research should explore the relation between these dietary factors and concentrations of biomarkers, including lipids, fatty acids, and inflammatory markers, to establish a better understanding of potential causal mechanisms.

This study was made possible by the contribution of many people, including the original investigators and the diligent team who recruited the participants and who continue working on follow-up. We thank the thousands of Melbourne residents who continue to participate in the study.

The authors’ responsibilities were as follows—LRH: design of analysis, statistical analysis, interpretation of results, tables, writing of manuscript, and funding; DRE: funding, interpretation of results, and commented on manuscript drafts; JP: design of original Melbourne Collaborative Cohort Study—(MCCS), interpretation of results, and commented on manuscript drafts; GGG: design and conduct of original MCCS and funding; AMT: funding, interpretation of results, and commented on manuscript drafts; AMH: design of analysis and commented on manuscript drafts; LB: design of analysis and commented on manuscript drafts; KO: design of original MCCS, funding, interpretation of results, and commented on manuscript drafts. None of the authors had a conflict of interest.

REFERENCES


Lymphocyte gene expression in subjects fed a low-choline diet differs between those who develop organ dysfunction and those who do not1–3

Mihai D Niculescu, Kerry-Ann da Costa, Leslie M Fischer, and Steven H Zeisel

ABSTRACT
Background: Some humans fed a low-choline diet develop hepatosteatosis, liver and muscle damage, and lymphocyte apoptosis. The risk of developing such organ dysfunction is increased by the presence of single-nucleotide polymorphisms (SNPs) in genes involved in folate and choline metabolism.

Objective: We investigated whether changes that occur in the expression of many genes when humans are fed a low-choline diet differ between subjects who develop organ dysfunction and those who do not. We also investigated whether expression changes were dependent on the presence of the SNPs of interest.

Design: Thirty-three subjects aged 20–67 y were fed for 10 d a baseline diet containing the recommended adequate intake of choline. They then were fed a low-choline diet for up to 42 d or until they developed organ dysfunction. Blood was collected at the end of each phase, and peripheral lymphocytes were isolated and used for genotyping and for gene expression profiling with the use of microarray hybridization.

Results: Feeding a low-choline diet changed the expression of 259 genes, and the profiles of subjects who developed and those who did not develop signs of organ dysfunction differed. Group clustering and gene ontology analyses found that the diet-induced changes in gene expression profiles were significantly influenced by the SNPs of interest and that the gene expression phenotype of the variant gene carriers differed significantly even with the baseline diet.

Conclusion: These findings support our hypothesis that a person’s susceptibility to organ dysfunction when fed a low-choline diet is modulated by specific SNPs in genes involved in folate and choline metabolism.

INTRODUCTION
Choline is an essential dietary nutrient involved in a multitude of metabolic roles (1). It is a major source of methyl groups for methionine synthesis and is needed for the structural integrity of cell membranes, the transport of lipids from the liver, and cholinergic neurotransmission (1). Adequate intake (AI) recommendations for choline have been established (2), and, in population studies, diets low in choline were associated with a greater risk of birth defects (3, 4) and with high homocysteine concentrations in blood (5). We previously reported that some humans fed a diet low in choline developed hepatosteatosis, experienced liver and muscle damage, and had greater lymphocyte apoptosis (6–10). All of these changes were reversed after a period of choline repletion. The susceptibility to these outcomes was not the same in all subjects; it varied with the presence of single-nucleotide polymorphisms (SNPs) within the genes involved in folate and choline metabolism (7, 8). With the notable exception of the carriers of the choline dehydrogenase (CHDH) variant allele (318 A→C; rs9001), who were protected against developing organ dysfunction (8), carriers of the methylenetetrahydrofolate dehydrogenase (MTHFD1) synthase variant (1958 G→A; rs2236225), the CHDH variant (432 G→T; rs12676), and the phosphatidylethanolamine methyltransferase (PEMT) variant (−744 G→C; rs12325817) alleles were at greater risk of developing organ dysfunction when fed a low-choline diet than were the carriers of the corresponding wild-type alleles (7, 8).

In this study, we fed humans a diet containing recommended amounts of choline, then fed the same subjects diets low in choline content, and then assessed gene expression in lymphocytes at the end of each feeding period. We determined whether ingesting a low-choline diet was associated with changes in gene expression, whether those subjects who developed organ dysfunction differed in gene expression from those who did not, and whether changes in gene expression were related to the presence of SNPs.

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SUBJECTS AND METHODS

Subjects

Healthy men (n = 31) and women (n = 35) were recruited for the study. Inclusion was contingent on an age-typical good state of health as determined by physical examination and standard clinical laboratory tests, such as complete blood count, blood chemistries, and fasting lipids and liver function tests and on the absence of known chronic diseases. Twenty-two subjects admitted to the study had minor elevations in blood lipids that were not deemed of clinical significance by the study physician. Of the originally recruited 66 subjects, 61 completed at least the initial and depletion phases. Of those 61 subjects, 1 was excluded because of a 9-kg weight loss during the study, 3 were excluded because they did not comply with diet restrictions, and 6 were excluded because they did not have baseline measurements; thus, 51 subjects were included in analyses. Of those subjects, 18 were excluded from the gene array analyses (see Microarray data analysis). The remaining 14 men and 19 women ranged in age from 20 to 67 y and had a body mass index (BMI; in kg/m²) between 19 and 31, which they maintained throughout the study. The ethnic distribution of these participants was 61% white, 30% African American, and 9% Asian, which reflects the local population characteristics of the Raleigh-Durham-Chapel Hill area.

Written informed consent was obtained from all participants. The study protocol was approved by the Institutional Review Board at the University of North Carolina at Chapel Hill (UNC-CH).

Study design

The participants were admitted to the UNC-CH General Clinical Research Center, where they remained under the supervision of study staff for the duration of the study. Research diets administered to the subjects, which were composed of 0.8 g high-biologic-value protein/kg body wt, with 30% of energy from fat and 70% of energy from carbohydrate, were prepared in-house to biologic-value protein/kg body wt, with 30% of energy from fat and 70% of energy from carbohydrate, were prepared in-house to protocol specifications and have been described in detail elsewhere (11). Total food intake was adjusted to be isocaloric and to provide adequate intakes of macronutrients and micronutrients. Initially, all participants received a diet of commonly eaten foods containing 550 mg choline/70 kg body wt⁻¹·d⁻¹—the presumed AI (2)—and 400 dietary folate equivalents (DFE)/d. The dietary choline content was confirmed as described previously (11), and the folate content was calculated by using the US Department of Agriculture SR16 database and PRONUTRA software (version 3.1.0.13; Viocare, Princeton, NJ). After 10 d of this baseline diet, liver fat was measured, and 48 mL blood was collected by venipuncture and processed for peripheral lymphocytes as described below. The subjects were randomly assigned to 2 groups—diet folate only or diet folate supplemented with 400 μg folic acid/d—and then were fed a diet in which the choline content was reduced to <50 mg/d, as confirmed by analysis of duplicate food portions. For the rest of the study, all diets offered to the diet-folate-only group contained 100 DFE/d, whereas the folic acid–supplemented group received an additional 668 DFE/d. Periodic measurements of urinary choline and betaine concentrations (12) were used to confirm compliance with the dietary restrictions. Subjects followed this depletion diet until they developed organ dysfunction associated with choline deficiency or for 42 d if they did not develop organ dysfunction. Blood (48 mL) was again collected at the end of the depletion phase. Humans were deemed to have organ dysfunction associated with choline deficiency if they had a >5-fold increase in serum creatine phosphokinase (CPK) activity (measurements were taken every 3–4 d) or if they had a >28% increase in liver fat content while following the choline-depletion diet (measurements were taken on days 21 and 42 of the choline-depletion diet) and if this increase in CPK activity or liver fat content was resolved when choline was returned to the diet. The change from baseline in liver fat content was estimated by using magnetic resonance imaging (MRI) in a clinical magnetic resonance system (Vision 41.5T; Siemens Medical Solutions, Malvern, PA) with a modified “In and Out of Phase” procedure that was described previously (13). Fat content was derived from measurements across 3–5 liver slices per subject and standardized to similarly measured slices of spleen.

Isolation of lymphocyte RNA and quality assessment

At the end of the baseline and choline-depletion diet phases, peripheral lymphocytes were isolated from blood within 2 h of collection by using Ficoll-Hypaque gradient in evacuated tubes with sodium citrate (Vacutainer CPT tubes; Becton Dickinson, Franklin Lakes, NJ), and suspended in TRIZol Reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated according to the manufacturer’s protocol, purified with the use of an RNeasy kit (Qiagen, Valencia, CA), and diluted to a standard concentration of 100 μg/mL. The subsequent procedures were performed at the UNC-CH Genomics Core Facility. Each sample (0.5 μg), including the reference RNA, was first tested on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) to ensure high-quality RNA before being diluted for hybridization.

Genotyping

Genomic DNA was prepared from peripheral blood with a commercial extraction kit (PureGene; Gentra Systems, Minneapolis, MN) according to the manufacturer’s instructions and diluted to a standard concentration of 1 μg/mL. The cytoplasmic MTHFD synthase (MTHFD1-G1958A) was amplified by multiplex polymerase chain reaction (PCR), purified, and then analyzed with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (7, 14). For the PEMT and CHDH genes, DNA sequencing was performed on double-stranded DNA templates obtained from genomic DNA by using PCR amplification (8).

Microarray hybridization and data retrieval

We mixed 12 μg of sample and an equal amount of universal human reference RNA (Stratagene, La Jolla, CA) with anchored oligo-dT Primer mixture, and amplified the mixture by using a thermal cycler (70 °C for 5 min to anneal; 42 °C for 1.5 h). The cDNA was then labeled in a reverse transcriptase reaction with dUTP CyDye–labeled nucleotides (Cy3 for reference and Cy5 for experiment) by using a CyScribe First-Strand cDNA Labeling Kit (GE Healthcare [formerly Amersham Biosciences], Piscataway, NJ). The labeled cDNA was then purified by degrading the mRNA with NaOH at 37 °C for 15 min and washing the samples with Tris-EDTA buffer by using a Microcon PCR filter (Millipore, Bedford, MA). The combined Cy3 + Cy5 sample was then applied to a glass slide array, sealed in a hybridization chamber, and incubated in a 65 °C water bath overnight. The human oligo-arrays with 16 000 spots were produced at the
Genomics Core Facility in the same batch by using 60-mer oligonucleotides (Compugen USA, Jamesburg, NJ). After hybridization, the array slides were washed and spun dry before being scanned to collect the fluorescent images (GenePix 4000B fluorescent scanner; Axon Instruments, Union City, CA). Images were gridded, and data were collected with the use of GENEPIX PRO microarray acquisition and analysis software (version 5.0; Axon Instruments). Detailed protocols are available at http://cancer.med.unc.edu/genomicscore/. Images obtained were analyzed by superimposing a grid for each array with the use of the GENEPIX PRO software. All spots of poor quality (as determined by visual inspection) were flagged as bad and removed from further analysis.

Microarray data analysis

All of the collected raw data files were further processed by uploading them into the UNC Microarray Database (https://genome.unc.edu/), and the data were filtered and retrieved according to the following criteria: 1) data were retrieved by the immutable Stanford University Identification (SUID) reference number to average the replicate spots by gene name and present the result as one (ie, to collapse them); 2) spots were selected only if they had both Channel 1 and Channel 2 Lowess-normalized means ≥30% above background; and 3) genes were selected only if they had >70% good data. No cutoffs were selected. Final data were expressed as log(base 2) of lymphocyte RNA-reference RNA Lowess normalized ratio (mean). The final number of arrays (and hence the number of subjects included in further analyses) was influenced by various criteria: not all RNA samples were of good quality, some blood samples did not contain enough RNA, and not all arrays passed the quality test. Therefore, of the 51 subjects initially included in the study, only 33 had arrays that were suitable for analysis. Of these 33, not all had 2 arrays (1 for baseline and 1 for depletion), so the number of arrays available at baseline was 30, and the number of arrays available at depletion was 25 (Table 1).

Table 1
Groups for analyses

<table>
<thead>
<tr>
<th>Analysis group</th>
<th>Symbol</th>
<th>Analysis group</th>
<th>Arrays</th>
<th>Genes changed</th>
</tr>
</thead>
<tbody>
<tr>
<td>All subjects</td>
<td>ALL SUBJECTS</td>
<td>Depletion vs Baseline</td>
<td>25vs30</td>
<td>259</td>
</tr>
<tr>
<td>Subjects with no organ dysfunction on low-choline diet</td>
<td>NO SIGNS</td>
<td>Depletion vs Baseline</td>
<td>12vs14</td>
<td>293</td>
</tr>
<tr>
<td>Subjects with organ dysfunction on low-choline diet</td>
<td>SIGNS</td>
<td>Depletion vs Baseline</td>
<td>13vs16</td>
<td>339</td>
</tr>
<tr>
<td>All subjects at depletion</td>
<td>ALL(D)</td>
<td>Signs vs No Signs</td>
<td>13vs12</td>
<td>416</td>
</tr>
<tr>
<td>PEMT(–744 G→C) group at baseline</td>
<td>PEMT(B)</td>
<td>SNP vs WT</td>
<td>22vs 8</td>
<td>357</td>
</tr>
<tr>
<td>PEMT(–744 G→C) group at depletion</td>
<td>PEMT(D)</td>
<td>SNP vs WT</td>
<td>19vs 6</td>
<td>441</td>
</tr>
<tr>
<td>PEMT(–744 G→C) group at depletion (women)</td>
<td>PEMT(W)</td>
<td>Women with SNP vs All WT</td>
<td>13vs 6</td>
<td>325</td>
</tr>
<tr>
<td>MTHFD1(–1958 G→A) group at baseline</td>
<td>MTHFD1 (B)</td>
<td>SNP vs WT at baseline</td>
<td>19vs11</td>
<td>558</td>
</tr>
<tr>
<td>MTHFD1(–1958 G→A) group at depletion</td>
<td>MTHFD1 (D)</td>
<td>SNP vs WT at depletion</td>
<td>17vs 8</td>
<td>409</td>
</tr>
<tr>
<td>CHDH(318 A→C) group at depletion</td>
<td>CHDH(A→C)</td>
<td>SNP vs WT at depletion</td>
<td>9vs16</td>
<td>450</td>
</tr>
<tr>
<td>CHDH(+432 G→T) group at depletion</td>
<td>CHDH(G→T)</td>
<td>SNP vs WT at depletion</td>
<td>9vs16</td>
<td>233</td>
</tr>
<tr>
<td>FOLATE group at depletion</td>
<td>FOLATE</td>
<td>Folate supplementation vs no supplementation at depletion</td>
<td>13vs12</td>
<td>360</td>
</tr>
</tbody>
</table>

1 ALL SUBJECTS, comparison of all subjects (depletion phase versus end of baseline); SIGNS, subjects who developed organ dysfunction when fed the low-choline diet; NO SIGNS subjects who did not develop organ dysfunction (end of depletion phase versus end of baseline); ALL(D), comparison of all subjects at the end of the low-choline diet (clinical signs versus absence of signs). For each polymorphism, single-nucleotide polymorphism (SNP) denotes the presence of the respective mutation, and WT is the wild-type genotype (see Introduction for genotype descriptions). The SNPs are indicated by the nucleotide position relative to the transcription starting site and by the nucleotide replacement. “(B)” after a gene symbol indicates the sample after the baseline (adequate choline) phase; “(D)” after a gene symbol indicates the sample after low-choline diet phase; “(W)” after a gene symbol indicates women only after the low-choline diet; FOLATE compares to subjects with and without folate supplementation at the end of the low-choline diet.

Statistical analysis of gene expression

Subjects were grouped for 12 comparisons (Table 1) according to the following criteria: presence or absence of organ dysfunction when fed a low-choline diet (hepatosteatosis and liver and muscle damage); timepoint (end of the baseline phase or end of the depletion phase); and the presence of a specific SNP for PEMT, MTHFD, and CHDH. Significance analysis of microarrays (SAM) (15) was applied to the final data by using TIGR MEV software [version 3; Dana-Farber Cancer Institute, Boston, MA (16)]. We used the one-class response type and 100 permutations or the maximum allowed permutations (if <100) to select the genes that are significantly changed across each of the groups. An arbitrary false discovery rate (FDR) of maximum 5% was chosen, and the closest threshold value (delta, or Δ) was selected for each group. The FDR is the expected percentage of false predictions; therefore, at 5% FDR, 95% of the observations are reproducible and not due to chance. The software generated a list of significantly overexpressed and underexpressed genes (d score assessment), and the q value (the lowest FDR at which the gene is called significant) was computed for each gene. The data generated by the use of SAM were converted accordingly for subsequent gene ontology (GO) classification: −1 for underexpression, 0 for no change, and 1 for overexpression. The defined groups (Table 1) were clustered according to their changes in gene expression (16). Each group was considered as an experiment, and gene changes were expressed as an average of all the arrays within the group.

Cluster analysis

The TIGR MEV software was used for cluster analysis, in which groups defined in Table 1 were clustered according to changes in gene expression (post-SAM analysis). Hierarchical clustering was assessed by using the average Euclidean distance between groups.
## Table 2

Selected genes with expression changes between baseline and depletion^1^  

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Score</th>
<th>ALL SUBJECTS (n = 25–30)</th>
<th>NO SIGNS group (n = 13–16)</th>
<th>SIGNS group (n = 12–14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALDH1A2</td>
<td>Aldehyde dehydrogenase 1 family, member A2</td>
<td>3.9</td>
<td>4.0</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>ARHGAP9</td>
<td>Rho GTPase–activating protein 9</td>
<td>−5.0</td>
<td>−4.5</td>
<td>−3.5</td>
<td></td>
</tr>
<tr>
<td>BUB1B</td>
<td>Budding uninhibited by benzimidazoles 1 homolog β</td>
<td>4.7</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>CCL2</td>
<td>Chemokine (C–C motif) ligand 2</td>
<td>4.0</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>CD163</td>
<td>CD163 molecule</td>
<td>−4.0</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>CD36</td>
<td>CD36 molecule (thrombospondin receptor)</td>
<td>−7.3</td>
<td>−8.5</td>
<td>−4.6</td>
<td></td>
</tr>
<tr>
<td>CDCA8</td>
<td>Cell division cycle–associated 8</td>
<td>4.1</td>
<td>4.2</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>CHEK1</td>
<td>CHK1 checkpoint homolog</td>
<td>−5.0</td>
<td>−7.2</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>COLEC12</td>
<td>Collectin subfamily member 12</td>
<td>4.7</td>
<td>4.6</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>C3T3</td>
<td>Cystatin C (amyloid angiopathy and cerebral hemorrhage)</td>
<td>−4.2</td>
<td>−3.7</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>CSTL</td>
<td>Cathepsin L</td>
<td>4.0</td>
<td>4.7</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>CX3CR1</td>
<td>Chemokine (C–X3–C motif) receptor 1</td>
<td>−8.5</td>
<td>−7.9</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>DOCK2</td>
<td>Dedicator of cytokinesis 2</td>
<td>−4.1</td>
<td>−4.2</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>FOXA1</td>
<td>Forkhead box A1</td>
<td>6.0</td>
<td>7.3</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>GBE1</td>
<td>Glucan (1,4–α), branching enzyme 1</td>
<td>4.2</td>
<td>−1.4</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>GZMK</td>
<td>Granzyme K (granzyme 3; trypase II)</td>
<td>−4.5</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>HSPA5</td>
<td>Heat shock 70-kDa protein 5 (glucose-regulated protein, 78 kDa)</td>
<td>4.0</td>
<td>4.9</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>HSPCB</td>
<td>Heat shock protein 90-kDa α (cytosolic), class B member 1</td>
<td>−4.4</td>
<td>=</td>
<td>−3.8</td>
<td></td>
</tr>
<tr>
<td>IL2RB</td>
<td>Interleukin 2 receptor β</td>
<td>−8.2</td>
<td>−7.8</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>KIF20A</td>
<td>Kinesin family member 20A</td>
<td>5.4</td>
<td>−1.1</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>KIF23</td>
<td>Kinesin family member 23</td>
<td>3.9</td>
<td>4.8</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>KLRF1</td>
<td>Killer cell lectin–like receptor subfamily F, member 1</td>
<td>−4.3</td>
<td>=</td>
<td>−3.8</td>
<td></td>
</tr>
<tr>
<td>MAL2</td>
<td>Mal, T cell differentiation protein 2</td>
<td>5.8</td>
<td>6.1</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>MARC1</td>
<td>Membrane-associated ring finger (C3HC4) 1</td>
<td>−4.3</td>
<td>=</td>
<td>−3.8</td>
<td></td>
</tr>
<tr>
<td>MGP</td>
<td>Matrix Gla protein</td>
<td>6.4</td>
<td>6.5</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>MMP2</td>
<td>Matrix metalloproteinase 2</td>
<td>4.7</td>
<td>4.8</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>MYOM2</td>
<td>Myomesin (M protein) 2, 165 kDa</td>
<td>−4.0</td>
<td>=</td>
<td>−4.0</td>
<td></td>
</tr>
<tr>
<td>NFKBIA</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor α</td>
<td>−4.1</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>PDAP1</td>
<td>PDGFA-associated protein 1</td>
<td>−4.4</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>PLAU</td>
<td>Plasminogen activator, urokinase receptor</td>
<td>−4.3</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>PRAME</td>
<td>Preferentially expressed antigen in melanoma</td>
<td>5.9</td>
<td>6.9</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>PTPRCA</td>
<td>Protein tyrosine phosphatase, receptor type, C-associated protein</td>
<td>4.4</td>
<td>=</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>RGS19</td>
<td>Regulator of G protein signaling 19</td>
<td>−4.6</td>
<td>−4.1</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>RNPEPL1</td>
<td>Arginyl aminopeptidase (aminopeptidase B)–like 1</td>
<td>−4.5</td>
<td>−3.7</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>SFPR1</td>
<td>Secreted frizzled-related protein 1</td>
<td>5.1</td>
<td>5.8</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Slc7a7</td>
<td>Solute carrier family 7 (cationic amino acid transporter, y+ system), member 7</td>
<td>−5.9</td>
<td>−5.6</td>
<td>−5.8</td>
<td></td>
</tr>
<tr>
<td>SMARCA1</td>
<td>SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 1</td>
<td>5.9</td>
<td>=</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
<td>4.7</td>
<td>3.8</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>TINFAP3</td>
<td>Tumor necrosis factor-α–induced protein 3</td>
<td>−4.3</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>UCK1</td>
<td>Uridine–cytidine kinase 1</td>
<td>5.1</td>
<td>5.6</td>
<td>=</td>
<td></td>
</tr>
</tbody>
</table>

^1^ Listed are the 20 most overexpressed and the 20 most underexpressed genes. Changes in the genes are presented as d scores. All d scores were calculated by using a delta value corresponding to a maximum false discovery rate of 5% for each comparison group. A positive d score indicates overexpression, and a negative d score indicates underexpression. ALL SUBJECTS, comparison of all subjects (depletion phase versus end of baseline); SIGNS, subjects who developed organ dysfunction when fed the low-choline diet; NO SIGNS, subjects who did not develop organ dysfunction (end of the depletion phase versus end of baseline).

^2^ No change according to significance analysis of microarrays (all such).

### Gene ontology classification

GOMINER software (version 1.22; Georgia Tech University, Atlanta, GA; Internet: http://discover.nci.nih.gov/gominer/) was used to construct a GO list of the significantly changed genes (17), by using gene symbols as identifiers. Data from the SAM output file were converted to text files and GOMINER generated a list of genes classified by their various GO classes, according to the default database (com.mysql.jdbc.Driver at jdbc:mysql://discover.nci.nih.gov/GEEVS). Fisher’s exact tests were performed to determine the significance of changes within the total number of genes in each GO class, and significance was separately assessed for the number of genes that were overexpressed, underexpressed, or both (P < 0.05).

### Gene expression validation

A small subset of genes was selected to determine the validity of the array-generated data. Real time reverse transcriptase (RT)-PCR was used to assess the expression of 4 of the most overexpressed genes and 4 of the most underexpressed genes. Primers for these 8
genes—FOXAI, PRAME, TERT, CDC8, CHEK1, IL2RB, TNFAIP3, and NFKBIA—were purchased from SuperArray (Frederick, MD) as was 18S rRNA, which was used as the normalization gene. Equal amounts of RNA were pooled from all subjects at baseline and depletion, respectively. For all genes but one, 100 ng pooled template RNA was used in quintuplicate reactions (one-step RT-PCR) in a QuantiTect SYBR Green RT-PCR kit (Qiagen); for 18S RNA, 10 ng template RNA was used. All reactions were performed on an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) under the following cycling conditions: reverse transcription for 30 min at 50 °C; initial activation for 15 min at 95 °C; and 45 cycles of denaturation for 15 s at 94 °C, annealing for 30 s at 55 °C, and extension for 30 s at 72 °C, which were followed by data acquisition for the melting curves. The differences and statistical assessment in gene expression were computed by using the comparative threshold (ΔΔCt) method with the REST384 add-in for EXCEL (Internet: http://www.gene-quantification.de/) (18), and gene expression changes were expressed as 18S-normalized ratios between depletion and baseline. Statistical significance of change was assessed by using both the Student t test and the nonparametric pairwise fixed reallocation randomization test provided by REST (18).

Regulation of gene expression by DNA methylation

We used the DNA Methylation Database (Internet: http://www.methdb.de/; last accessed 12/05/2006) to compare genes found to be changed by dietary choline with those reported to have their expression regulated by DNA methylation (19).

RESULTS

Changes in gene expression between baseline and depletion

Of the 12,034 unigenes present within the arrays, 152 were underexpressed, and 107 were overexpressed in subjects after the low-choline diet phase of the study. A list of selected genes (the 20 most overexpressed and the 20 most underexpressed) is shown in Table 2. When subjects were further subclassified according to the absence or presence of signs of organ dysfunction (fatty liver, increased plasma CPK, or both) after being fed a low-choline diet, the gene expression profiles of the 2 groups could be differentiated (Table 2). From the ALL SUBJECTS group, we chose a limited number of genes to validate the array data by real-time RT-PCR (Table 3). With the exception of CHEK1, changes in gene expression, given as the relative ratio between depletion and baseline, were confirmed as significant by one or both of the statistical tests (t test and the pairwise fixed reallocation randomization test).

Hierarchical clustering between defined groups

Using the data obtained from SAM analysis, we clustered the groups defined in Table 1 to determine whether specific changes in gene expression profiles were associated with the presence of signs of organ dysfunction when subjects were following a low-choline diet, with the presence of PEMT, CHDH, and MTHFD1 SNPs, or both. In addition, we investigated whether gene expression profiles at baseline were different between these groups (which would suggest a different phenotype at baseline). The tree node of the groups, each one being considered an experiment, is shown in Figure 1. According to the node distribution, 2 main branches were generated for all the experiments. One branch included the PEMT and MTHFD1 groups surrounding the group with all subjects at the end of the depletion phase [ALL(D)]. The other main branch includes both CHDH polymorphisms, and the groups based on the presence (SIGNS) or the absence (NO SIGNS) of signs of organ dysfunction, all centered around the main ALL SUBJECTS group. The ALL SUBJECTS, SIGNS, and NO SIGNS groups were used to compare gene expression at the end of the depletion phase and the end of the baseline phase. ALL(D) compared the SIGNS group with the NO SIGNS group at the end of the depletion period. Four clusters were identified with the smallest distance: 1) subjects with the PEMT SNP fed a low-choline diet [PEMT(D)] associated with women with the PEMT SNP fed a low-choline diet [PEMT(W)]; 2) ALL(D) associated with subjects with the MTHFD1 SNP fed a low-choline diet [MTHFD1(D)]; 3) ALL SUBJECTS associated with subjects who developed organ dysfunction when fed a low-choline diet (SIGNS); and 4) one of the CHDH SNPs [CHDH(G→T)] associated with the FOLATE group.

Changes in gene expression across all groups

When the SAM analysis was performed for all groups defined in Table 1, changes for 1054 unigenes were significant. A complete list of these genes is available at http://www.unc.edu/zeisel_lab/. On the basis of our GO analysis, we present data for a limited number of genes (Figure 2) that are included in the following GO classes of special interest for our human study: folate-thymidine metabolism (GO 19860, 42083, and 46104), apoptosis (GO 6915, 6916, 6917, 8632, 30693, 43027, 43065, and 43154), DNA damage-repair (GO 77, 724, 725, 31570, 42770, and 51908), cell cycle regulation-proliferation (GO 4861 and 8283), immune-inflammatory response and lymphocyte

Table 3

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) validation of the gene array data

<table>
<thead>
<tr>
<th>FOXAI</th>
<th>PRAME</th>
<th>TERT</th>
<th>CDC8</th>
<th>CHEK1</th>
<th>IL2RB</th>
<th>TNFAIP3</th>
<th>NFKBIA</th>
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<td>11.03</td>
<td>2.15</td>
<td>4.84</td>
<td>0.81</td>
<td>0.67</td>
<td>0.61</td>
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<td>± SE</td>
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<td>± 2.25</td>
<td>± 0.36</td>
<td>± 0.81</td>
<td>± 0.10</td>
<td>± 0.14</td>
<td>± 0.07</td>
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<tr>
<td>t Test</td>
<td>*2</td>
<td>*</td>
<td>*</td>
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<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
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<td>*</td>
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<td>4.7</td>
<td>4.1</td>
<td>-5.0</td>
<td>-8.2</td>
<td>-4.3</td>
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</tbody>
</table>

1 SAM, significance analysis of microarrays. Gene expression data from the microarray study was compared with real-time RT-PCR for 8 of the genes indicated in Table 1 (ALL SUBJECTS analysis). 2 Significant value, P < 0.05. 3 A positive score indicates overexpression, and a negative score indicates underexpression per SAM analysis.
differentiation-activation (GO 1772, 6952, 6955, 19731, 19732, 19864, 30098, 42113, 42742, and 50832), epigenetic regulation (GO 8327, 35035, and 45815), and telomere maintenance (GO 3720, 3721, 5697, and 42162).

Gene ontology analysis

GO analysis was performed for all genes deemed significantly changed within any of the groups defined in Table 1. Fisher’s exact test indicated either unidirectional (up or down) or bidirectional (both up and down) changes for 1216 GO classes (a complete list of changes within GO classes is available at http://www.unc.edu/zeisel_lab/). Changes across selected GO classes listed above are shown in Table 4, where the vast majority of changes are in one direction, which suggests that, within each GO class, changes in gene expression are functionally convergent.

Genes regulated by DNA methylation

Previous reports in cell culture and animal model systems show that dietary choline modulates gene expression by altering DNA methylation (20, 21), but no data are yet available in humans fed a low choline diet. Using the only available public database on DNA methylation (see Subjects and Methods), we identified 13 of the 1024 genes changed in our study that were reported to be regulated by DNA methylation in various studies using human tissues or human cells in culture (Table 5).

DISCUSSION

We found that a low-choline diet induced changes in lymphocyte gene expression in humans. These changes included (but were not limited to) changes in expression of genes functionally involved in folate metabolism, apoptosis, DNA damage-repair, cell cycle regulation, immune response, epigenetic regulation, and telomere maintenance, which suggested that dietary choline deficiency can alter the functionality of many pathways. Subjects who developed organ dysfunction while following a low-choline diet differed from those who did not develop organ dysfunction in their expression of many genes, including some of those related to any or all of apoptosis, the DNA integrity checkpoint, and genes of cell cycle regulation. Subjects with the PEMT (rs12325817) and MTHFD1 (rs2236225) SNPs, previously shown to predispose a person to developing organ dysfunction when fed a low-choline diet (7, 8), differed at baseline from those subjects without the SNP in their expression of apoptosis, the DNA damage checkpoint, and cell proliferation control genes, which suggests that they are phenotypically different even before a low-choline diet is administered.

In different comparison groups, choline deficiency induced different patterns of change (Figure 1). Moreover, many of the results reported within all subjects (baseline compared with depletion, ALL SUBJECTS) could be misleading because these changes were not homogenous when subjects were classified on the basis of signs of organ dysfunction (Table 2 and Figure 2). For instance, 3 of the genes with the largest change in expression (CHEK1, GBE1, and KIF20A) were differently expressed in the NO SIGNS group than in the SIGNS group (Table 2). CHEK1 is involved in DNA repair in human T lymphocytes (22). GBE1 is required for sufficient glycogen accumulation and is normally underexpressed in whole human blood (23). KIF20A regulates the transport of Golgi membranes and associated vesicles along microtubules (24).
By clustering the groups according to genotype (Figure 1), we found that different patterns in gene expression do indeed support this classification. For example, the previously reported (8) protective CHDH (318 A→C) genotype grouped close to the NO SIGNS group—those who had no clinical symptoms while following the low-choline diet—whereas the CHDH (432

FIGURE 2. Gene expression profiles across all groups. The computed change in expression is shown for each gene included in gene ontology (GO) classes pertaining to folate-thymidine metabolism, apoptosis, DNA damage-repair, cell cycle regulation-proliferation, immune-inflammatory response and lymphocyte differentiation-activation, epigenetic regulation, and telomere maintenance. ■, overexpression; □, no change; △, underexpression within each comparison group (by significance analysis by microarray; the false discovery rate was <5%) as indicated in Table 1. ALL SUBJECTS, comparison of all subjects (the end of the depletion phase compared with the end of the baseline phase); SIGNS, the subjects who developed organ dysfunction when fed the low-choline diet; NO SIGNS, the subjects who did not develop organ dysfunction (comparing the end of the depletion phase with the end of the baseline phase). For each polymorphism, the gene symbol is indicated. The choline dehydrogenase single-nucleotide polymorphisms are indicated by the nucleotide replacement, and the sample is at the end of depletion phase. The notation “(B)” after the gene symbol indicates the sample after the baseline (adequate choline) phase; “(D)” after the gene symbol indicates the sample after the low-choline diet phase; “(W)” after the gene symbol indicates women only after the depletion phase. FOLATE compares subjects with and without folate supplementation at the end of the depletion phase. The number of arrays for each comparison is provided in Table 1.
Table 4
Gene expression changes in selected gene ontology (GO) groups

<table>
<thead>
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<th>GO Identifier</th>
<th>GO class</th>
<th>ALL SUBJECTS</th>
<th>ALL (D)</th>
<th>NO SIGNS</th>
<th>SIGNS</th>
<th>PEMT (D)</th>
<th>PEMT (B)</th>
<th>PEMT (W)</th>
<th>MTHFD1 (D)</th>
<th>MTHFD1 (B)</th>
<th>CHDH (A→C)</th>
<th>CHDH (G→T)</th>
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</tbody>
</table>

1 GO analysis was used to assess the significance of change for genes according to their GO class (see Materials and Methods). All analysis groups are as defined in Table 1. ALL SUBJECTS, comparison of all subjects (depletion phase versus end of baseline); NO SIGNS, subjects who did not develop organ dysfunction (end of depletion phase versus end of baseline). For each polymorphism, the gene symbol is given. The choline dehydrogenase (CHDH) single-nucleotide polymorphisms are indicated by the nucleotide replacement, and the sample is at the end of the depletion phase. The notation “(B)” after the gene symbol indicates sample after the baseline (adequate choline) phase; “(D)” after the gene symbol indicates sample after the low-choline diet phase; “(W)” after the gene symbol indicates women only after the depletion phase. FOLATE compares subjects with and without folate supplementation at the end of the depletion phase. The number of arrays for each comparison is specified in Table 1. Fisher’s exact test was used to assess significance of change within each GO class within genes that are overexpressed, underexpressed, or both. Up arrows denote significance of the number of genes that were overexpressed; down arrows denote significance for the number of the underexpressed genes, whereas both arrows indicate significance for the total number of both overexpressed and underexpressed genes within each GO class (P < 0.05). An equals sign denotes no change.

G→T) genotype, which was reported to increase susceptibility to choline deficiency (8), grouped with the SIGNS group. Moreover, the NO SIGNS and SIGNS groups were intercalated by the ALL SUBJECTS group, which supports the heterogeneity of the previously reported responses to dietary choline deficiency. Included in the other arm from the clustering analysis were the PEMT and MTHFD1 genotypes, which grouped around the ALL(D) group. This cluster supported our hypothesis that genetic differences account for the presence or absence of organ dysfunction in humans depleted of choline (7, 8). This analysis also found that the presence of the PEMT and MTHFD1 genotypes could confer differences in the phenotypes at baseline, which suggests that different persons may have different susceptibility to dietary choline deficiency and that the risk of choline
involved in apoptosis (Table 4), in which the alleles (Table 4). A different pattern was observed for genes developed organ dysfunction when fed a choline-deficient diet consistent with our previous data, which showed that humans who in negative regulation of caspase activity). This result is consis-
tent, perhaps by epigenetic changes in gene expression. The folate status of subjects had little effect on the gene expression changes seen in GO analyses.

Some genes that are regulated by DNA methylation were also identified as being changed by a low-choline diet (Table 5). In cultured human neuroblastoma cells and in rodent models, choline deficiency alters both global and gene-specific DNA methylation and the expression of these genes (20, 21). Therefore, we suggest that the observed choline deficiency–induced changes in gene expression occurred because of altered methylation in promoter regions of the genes involved. Among the genes changed in choline deficiency that are known to be regulated by gene methylation, the insulin-like growth factor 2 (IGF2) is an imprinted gene; loss of imprinting is associated with cancer in various experimental models (as reviewed in reference 26). Another gene identified as being changed by dietary choline and known to be regulated by methylation is telomerase reverse transcriptase (TERT), the product of which is the protein component of a ribonucleoprotein polymerase that maintains telomere ends by addition of the telomere repeat TTAGGG, and its deregulation is involved in both cellular senescence (telomere shortening) and carcinogenesis in leukemic cells (27).

In conclusion, dietary choline deficiency induced changes in gene expression profiles in human lymphocytes, and these patterns correlated with the occurrence of organ dysfunction and apoptosis in humans fed a low-choline diet. These outcomes also correlated with polymorphisms in genes that regulate folate and choline metabolism. Further studies are required to determine whether these changes are regulated by epigenetic mechanisms and to identify other populations at risk for dietary choline deficiency.

deficiency is greater in women who are carriers of the PEMT allele [this group was closer to the ALL(D) group than to the PEMT(D) group]. Harder to interpret is the unexpected clustering of the FOLATE group (those receiving folate supplements versus those who did not) close to the CHDH (432 G→T) genotype.

To construct a better picture of the potential functional significance of these gene expression patterns, we used GO analysis to group genes according to their functional roles. The heterogeneity of the response to a low choline diet was also shown at this level of analysis. Genes involved in folate metabolism were most affected in the carriers of the CHDH (432 G→T) single-nucleotide polymorphisms are indicated by the nucleotide replacement, and the sample is at the end of depletion phase. The annotation “(B)” after a gene symbol indicates the sample after the baseline (adequate choline) phase; “(D)” after a gene symbol indicates the sample after the low-choline diet phase; “(W)” after a gene symbol indicates women only after the depletion phase. FOLATE compares subjects with and without folate supplementation at the end of the depletion phase. The number of arrays for each comparison is specified in Table 1.

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Table 5
Genes of which the expression is changed by a low-choline diet and that are known to be regulated by DNA methylation

<table>
<thead>
<tr>
<th>Gene</th>
<th>ALL SUBJECTS</th>
<th>ALL (D)</th>
<th>NO SIGNS</th>
<th>SIGNS</th>
<th>PEMT (D)</th>
<th>PEMT (B)</th>
<th>PEMT (W)</th>
<th>MTHFD1(D)</th>
<th>MTHFD1(B)</th>
<th>CHDH (A→C)</th>
<th>CHDH (G→T)</th>
<th>FOLATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASP8</td>
<td>Up</td>
<td>Down</td>
<td>Down</td>
<td>Up</td>
<td>Down</td>
<td>Down</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COL6A1</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Down</td>
<td>Down</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CSPG2</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Down</td>
<td>Down</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSCR5</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Down</td>
<td>Down</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAGE2</td>
<td>Up</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IFNA8</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Down</td>
<td>Down</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF2</td>
<td>Up</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
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<td></td>
</tr>
<tr>
<td>PFKL</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
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<td></td>
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<tr>
<td>RASSF1</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
<td>Down</td>
<td>Down</td>
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<td></td>
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<tr>
<td>RCN1</td>
<td>Up</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
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<td></td>
<td></td>
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<tr>
<td>TERT</td>
<td>Up</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TFF1</td>
<td>Up</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP3</td>
<td>Down</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

1“Up” and “Down” indicate the direction of change in gene expression. Within all genes changed in the comparison groups defined in Table 1, 13 genes were found to be regulated by DNA methylation according to a search with the DNA Methylation Database (see Materials and Methods). ALL SUBJECTS, comparison of all subjects (depletion phase versus end of baseline); NO SIGNS, subjects who developed organ dysfunction when fed the low-choline diet; NO SIGNS, subjects who did not develop liver dysfunction (end of depletion phase versus end of baseline). For each polymorphism, the gene symbol is given. The choline dehydrogenase (CHDH) single-nucleotide polymorphisms are indicated by the nucleotide replacement, and the sample is at the end of depletion phase.

To construct a better picture of the potential functional significance of these gene expression patterns, we used GO analysis to group genes according to their functional roles. The heterogeneity of the response to a low choline diet was also shown at this level of analysis. Genes involved in folate metabolism were most affected in the carriers of the CHDH (432 G→T) single-nucleotide polymorphisms are indicated by the nucleotide replacement, and the sample is at the end of depletion phase. The annotation “(B)” after a gene symbol indicates the sample after the baseline (adequate choline) phase; “(D)” after a gene symbol indicates the sample after the low-choline diet phase; “(W)” after a gene symbol indicates women only after the depletion phase. FOLATE compares subjects with and without folate supplementation at the end of the depletion phase. The number of arrays for each comparison is specified in Table 1.

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The authors’ responsibilities were as follows—SHZ (principal investigator): designed the human study, oversaw the conduct of the study, and participated in data interpretation and manuscript preparation; LMF: supervised the recruitment of subjects and the conduct of the human diet study; MDN: participated in data interpretation and manuscript preparation; LMF: supervised the recruitment of subjects and the conduct of the human diet study; KD (study coordinator): responsible for sample collection and processing, RNA extraction, quality assurance, and microarray hybridization; and all authors: participated in the writing of the manuscript. SHZ serves on advisory boards for the DuPont Company, the Solae Company, and Hershey Foods; LMF is a consultant with Hannaford Brothers Company. None of the authors had a personal or financial conflict of interest.

REFERENCES

Genetic polymorphism of the adenosine A$_{2A}$ receptor is associated with habitual caffeine consumption$^{1-3}$

Marilyn C Cornelis, Ahmed El-Sohemy, and Hannia Campos

**ABSTRACT**

**Background:** Caffeine is the most widely consumed stimulant in the world, and individual differences in response to its stimulating effects may explain some of the variability in caffeine consumption within a population.

**Objective:** We examined whether genetic variability in caffeine metabolism [cytochrome P450 1A2 (CYP1A2) $\sim$163A→C] or the main target of caffeine action in the nervous system [adenosine A$_{2A}$ receptor (ADORA2A) 1083C→T] is associated with habitual caffeine consumption.

**Design:** Subjects ($n =$ 2735) were participants from a study of gene-diet interactions and risk of myocardial infarction who did not have a history of hypertension. Genotype frequencies were examined among persons who were categorized according to their self-reported daily caffeine intake, as assessed with a validated food-frequency questionnaire.

**Results:** The ADORA2A, but not the CYP1A2, genotype was associated with different amounts of caffeine intake. Compared with persons consuming $<100$ mg caffeine/d, the odds ratios for having the ADORA2A TT genotype were 0.74 (95% CI: 0.53, 1.03), 0.63 (95% CI: 0.48, 0.83), and 0.57 (95% CI: 0.42, 0.77) for those consuming 100–200, >200–400, and $>$400 mg caffeine/d, respectively. The association was more pronounced among current smokers than among nonsmokers ($P$ for interaction = 0.07). Persons with the ADORA2A TT genotype also were significantly more likely to consume less caffeine (ie, $<100$ mg/d) than were carriers of the C allele ($P = 0.011$ (nonsmokers), $P = 0.008$ (smokers)).

**Conclusion:** Our findings show that the probability of having the ADORA2A 1083TT genotype decreases as habitual caffeine consumption increases. This observation provides a biologic basis for caffeine consumption behavior and suggests that persons with this genotype may be less vulnerable to caffeine dependence. *Am J Clin Nutr* 2007;86:240–4.

**KEY WORDS** Caffeine, ADORA2A, adenosine A$_{2A}$ receptor gene, CYP1A2, cytochrome P450 1A2, genotype, epidemiology, dependence

**INTRODUCTION**

Caffeine is the most widely consumed stimulant in the world with an estimated 80–90% of adults reporting regular consumption of caffeine-containing beverages and foods (1). Caffeine intakes vary widely from country to country and from person to person (2, 3). The pleasurable and reinforcing effects of caffeine have led to some concern that it is a potential drug of dependence (1, 4, 5). However, some persons experience anxiety, tachycardia, nervousness, or other adverse effects with low-to-moderate intakes of caffeine (4). These differences in response to caffeine may explain some of the variability in caffeine intake within a population (1, 6, 7). Although demographic, psychosocial, health-related, and environmental factors such as smoking have been linked to habitual caffeine consumption (8–11), there is some evidence that genetic factors are also important (12–15). Twin studies report heritability estimates of up to 77% for caffeine use, toxicity, tolerance, and withdrawal symptoms (12–15), but the specific genes involved are not yet identified.

Caffeine is metabolized primarily by cytochrome P450 1A2 (CYP1A2) in the liver through an initial N$^\circ$-demethylation (16, 17). CYP1A2 accounts for $\approx$95% of caffeine metabolism and shows wide variability in enzyme activity between persons (17–19). An A to C substitution at position −163 (rs762551) in the CYP1A2 gene decreases enzyme inducibility as measured by plasma or urinary caffeine-to-metabolite ratios after a dose of caffeine (20). Carriers of the $-163C$ allele can be considered slow caffeine metabolizers, whereas persons who are homozygous for the $-163A$ allele are more rapid caffeine metabolizers (20). It is not clear, however, whether CYP1A2 genotype alters caffeine consumption.

In amounts typically consumed from dietary sources, caffeine antagonizes the actions of adenosine at the adenosine A$_{2A}$ receptor (1), which was shown to play an important role in the stimulating and reinforcing properties of caffeine (21, 22). A$_{2AR}$ knockout mice have been found to have less of an appetite for caffeine than do their wild-type littermates (23). A C-to-T substitution at nucleotide position 1083 (rs751876) (also referred to as 1976C→T) in the ADORA2A gene, which codes for the A$_{2A}$

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receptor, was associated with caffeine-induced anxiety among nonhabitual caffeine consumers (24). Persons who were homozygous for the 1083T allele experienced greater anxiety after consuming 150 mg caffeine (24). However, it is not known whether persons with that genotype limit their habitual caffeine intake because of such adverse physiologic effects. The purpose of the present study was to examine whether genetic variability in caffeine metabolism (ie, CYP1A2) or the major target of caffeine action in the central nervous system (CNS) (ie, ADORA2A) is associated with habitual caffeine consumption in a free-living population.

SUBJECTS AND METHODS

Study design and participants

Details of the study design (case-control study) and participants were reported previously (25). Subjects were self-described Hispanic Americans living in Costa Rica and participating in a study of gene-diet interactions and risk of myocardial infarction (MI). Eligible cases were men and women who were survivors of a first acute MI between 1994 and 2004. Cases were ineligible if they died during hospitalization, were ≥75 y old on the day of their first MI, were physically or mentally unable to answer the questionnaire, or had a previous hospital admission related to cardiovascular disease. One control for each case, matched for age (±5 y), sex, and area of residence (county), was randomly selected with the use of information available at the National Census and Statistics Bureau of Costa Rica. Because of the comprehensive social services provided in Costa Rica, all persons living in the catchment areas had access to medical care without regard to income. Controls were ineligible if they were physically or mentally unable to answer the questionnaires or if they had a previous hospital admission related to MI or other cardiovascular disease. Participation for eligible cases and controls was 98% and 88%, respectively. For the current study, all subjects reporting a history of hypertension were excluded because these persons may have reduced their caffeine intake on the advice of their physician. Indeed, a significantly (P < 0.001) smaller proportion of persons with a history of hypertension (14%) than of persons with no history of hypertension (21%) reported consuming >400 mg caffeine/d. All subjects were visited at their homes for the collection of information on diet and medical history, for anthropometric measurements, and collection of biologic specimens.

Cases and controls gave written informed consent. The study was approved by the ethics committees of the Harvard School of Public Health and the University of Costa Rica, the Office of Protection from Research Risk at the National Institutes of Health, and the ethics review committee at the University of Toronto.

All data were collected during an interview with trained fieldworkers who used 2 questionnaires. The questionnaires consisted of closed-ended questions about smoking, sociodemographic characteristics, socioeconomic status, physical activity, diet, and medical history, including use of medication and personal history of diabetes and hypertension. Dietary intake was collected with the use of a 135-item semiquantitative food-frequency questionnaire specifically developed and validated to assess dietary intake during the previous year in the Costa Rican population (26). For cases, average intake represented the year preceding their MI. Included in the food-frequency questionnaire were questions about the consumption of caffeinated coffee, tea, cola beverages, and chocolate. Total caffeine intake was calculated with the use of the US Department of Agriculture food-composition data file. Subjects were categorized into 4 groups with self-reported caffeine intakes of <100, 100–200, >200–400, or >400 mg/d.

Genotyping

Blood samples were collected in the morning at the subject’s home after an overnight fast and were centrifuged at 1430 × g for 4 min at 20 °C to separate the plasma and leukocytes for DNA isolation by standard procedures. The CYP1A2 −163A→C (rs762551) and ADORA2A 1083C→T (rs5751876) polymorphisms were detected by restriction-fragment length polymorphism–polymerase chain reaction as described previously (27, 28). Genotype distributions among subjects did not deviate from Hardy-Weinberg equilibrium (P > 0.05).

Statistical analysis

All data were analyzed with the use of SAS software (version 8.2; SAS Institute, Cary, NC). DNA was available from 2873 subjects with no history of hypertension. Because caffeine consumption data were based on the year before incidence of MI, cases with nonfatal MI as well as population-based controls were included in the analyses. Nine subjects with missing data on caffeine intake and smoking status and 129 who could not be genotyped for either CYP1A2 or ADORA2A were also excluded from the study. These exclusions left a total sample size of 2735 for the final analyses.

Significant differences in the distribution of lifestyle characteristics by CYP1A2 and ADORA2A genotype were tested with the use of Pearson’s chi-square test (categorical variables) or t tests (continuous variables). Analyses were conducted with the use of a dominant CYP1A2 C allele model with AC and CC genotypes (slow metabolizers) combined, because the 2 groups have a similar rate of caffeine metabolism (20). For ADORA2A, results are presented with the use of a recessive ADORA2A T allele model with CC and CT genotypes combined because no differences in caffeine-induced anxiety were reported between persons with the CC or CT genotype (24). Odds ratios and 95% CIs were estimated by unconditional logistic regression to determine the relation between caffeine consumption and the risk of having the CYP1A2 C allele or ADORA2A TT genotype with the lowest caffeine intake (<100 mg/d) as the reference group. A test for linear trend was calculated across categories of caffeine intake for each polymorphism by treating caffeine intake as an ordinal variable. Pearson’s chi-square test with 1 df was used to compare the proportion of light caffeine consumers (ie, persons consuming <100 mg caffeine/d) among each genotype. Non-smokers (never or past smokers) and current smokers were examined separately because smokers metabolize caffeine more rapidly than non-smokers, and smokers may respond differently to the stimulating effects of caffeine as a result of the interaction of the A2A receptor with the dopamine D2 receptor, which plays a role in the behavioral effects of both caffeine and nicotine (1). Caffeine-smoking interactions were tested by comparing −2 log (likelihood) ratios from a model with caffeine intakes and smoking as main effects only and from another that included their interaction term. All statistical analysis were 2-sided, and P values < 0.05 were considered significant.
Table 1: Subject characteristics by cytochrome P4501A2 (CYP1A2) and adenosine A2A receptor (ADORA2A) genotype

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CYP1A2 = 163A → C</th>
<th>ADORA2A = 1083C → T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA (n = 1241)</td>
<td>AC (n = 1214)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>57.0 ± 11.22</td>
<td>56.8 ± 11.7</td>
</tr>
<tr>
<td>Male (%)</td>
<td>79</td>
<td>81</td>
</tr>
<tr>
<td>Urban residence (%)</td>
<td>74</td>
<td>74</td>
</tr>
<tr>
<td>Smoking status (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never or past smoker</td>
<td>65</td>
<td>63</td>
</tr>
<tr>
<td>1–9 cigarettes/d</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>≥10 cigarettes/d</td>
<td>25</td>
<td>28</td>
</tr>
<tr>
<td>Current alcohol consumption (%)</td>
<td>52</td>
<td>56</td>
</tr>
<tr>
<td>Income (US$/mo)</td>
<td>528 ± 401</td>
<td>543 ± 405</td>
</tr>
<tr>
<td>Secondary education or higher (%)</td>
<td>39</td>
<td>41</td>
</tr>
<tr>
<td>Physical activity (METs)</td>
<td>1.58 ± 0.76</td>
<td>1.62 ± 0.75</td>
</tr>
<tr>
<td>History of diabetes (%)</td>
<td>13</td>
<td>12</td>
</tr>
</tbody>
</table>

Note: Values are mean ± SD unless otherwise specified.

Table 2: Odds ratio of having the cytochrome P4501A2 (CYP1A2) = 163C allele for caffeine intake among nonsmokers and current smokers

<table>
<thead>
<tr>
<th>CYP1A2 genotype</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>AC + CC</td>
</tr>
<tr>
<td>All subjects</td>
<td></td>
</tr>
<tr>
<td>&lt;100 mg/d</td>
<td>108 (43)</td>
</tr>
<tr>
<td>100–200 mg/d</td>
<td>190 (49)</td>
</tr>
<tr>
<td>&gt;200–400 mg/d</td>
<td>694 (46)</td>
</tr>
<tr>
<td>&gt;400 mg/d</td>
<td>249 (42)</td>
</tr>
<tr>
<td>P for trend</td>
<td>0.38</td>
</tr>
<tr>
<td>Nonsmokers</td>
<td></td>
</tr>
<tr>
<td>&lt;100 mg/d</td>
<td>91 (44)</td>
</tr>
<tr>
<td>100–200 mg/d</td>
<td>146 (47)</td>
</tr>
<tr>
<td>&gt;200–400 mg/d</td>
<td>472 (47)</td>
</tr>
<tr>
<td>&gt;400 mg/d</td>
<td>104 (42)</td>
</tr>
<tr>
<td>P for trend</td>
<td>0.80</td>
</tr>
<tr>
<td>Current smokers</td>
<td></td>
</tr>
<tr>
<td>&lt;100 mg/d</td>
<td>17 (38)</td>
</tr>
<tr>
<td>100–200 mg/d</td>
<td>44 (56)</td>
</tr>
<tr>
<td>&gt;200–400 mg/d</td>
<td>222 (44)</td>
</tr>
<tr>
<td>&gt;400 mg/d</td>
<td>145 (42)</td>
</tr>
<tr>
<td>P for trend</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Results were determined by unconditional logistic regression.
intake. However, we found no association between the CYP1A2 −163A→C polymorphism and caffeine intake. This is consistent with our previous study showing no differences in CYP1A2 genotype frequencies across categories of coffee intake (25). Although coffee is the main source of caffeine in this population (>90% of total caffeine intake), our previous study included subjects with a history of hypertension who may have been avoiding caffeine because of its link with high blood pressure (29). These observations suggest that, for caffeine consumption behavior, persons may not be sensitive to differences in the rate of caffeine metabolism, but they appear to be sensitive to differences in the interaction between caffeine and the adenosinergic system.

Previous studies have identified numerous environmental factors that are associated with caffeine consumption, many of which have been accounted for in observational studies of caffeinated beverage consumption and various health outcomes. Because our findings suggest that the ADORA2A 1083C→T polymorphism is associated with caffeine consumption within a population, this polymorphism may be a potential genetic confounder in these observational studies.

A2A receptor–mediated adenosinergic neuromodulation was implicated in the development of various neurologic disorders, such as Parkinson’s disease, schizophrenia, and panic disorder. Studies have examined the association between the ADORA2A 1083C→T polymorphism and the risk of these disorders (30, 31), but findings have been inconsistent. On the basis of Mendel’s principle of independent inheritance, these studies reasonably assume that the ADORA2A 1083C→T polymorphism is a marker of A2A receptor function, which is unlikely to be associated with diet or other lifestyle characteristics (32). Therefore, any difference in risk should provide evidence for the role of the A2A receptor in the development of these disorders. Although ADORA2A genotype may reflect A2A receptor function, our findings show that it is also associated with caffeine consumption, thereby violating the assumption of independence. As a result, caffeine consumption may be a confounder in studies examining the main effect of ADORA2A genotype on various health outcomes.

Debate is ongoing as to whether caffeine is a potential drug of dependence (1, 4). The 10th edition of the International Statistical Classification of Diseases and Related Health Problems from the World Health Organization recognizes a diagnosis of substance dependence due to caffeine, but the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders from the American Psychiatric Association does not. Caffeine elicits pleasurable and reinforcing effects in some persons that may lead to dependence (1, 4, 5). Other persons, however, experience anxiety, tachycardia, nervousness, or other adverse effects with low-to-moderate intakes of caffeine, and they are unlikely to develop dependence (1, 4). A polymorphism of the ADORA2A gene was previously associated with caffeine-induced anxiety (24), and we now show that persons with this genotype limit their

### TABLE 3
Odds ratio of having the adenosine A2A receptor (ADORA2A) 1083TT genotype for caffeine intake among nonsmokers and current smokers

<table>
<thead>
<tr>
<th>Caffeine intake</th>
<th>ADORA2A genotype</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All subjects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100 mg/d</td>
<td>150 (60)</td>
<td>100 (40)</td>
</tr>
<tr>
<td>100–200 mg/d</td>
<td>261 (67)</td>
<td>129 (33)</td>
</tr>
<tr>
<td>&gt;200–400 mg/d</td>
<td>1062 (70)</td>
<td>446 (30)</td>
</tr>
<tr>
<td>&gt;400 mg/d</td>
<td>426 (73)</td>
<td>161 (27)</td>
</tr>
<tr>
<td>P for trend</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmokers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100 mg/d</td>
<td>127 (62)</td>
<td>78 (38)</td>
</tr>
<tr>
<td>100–200 mg/d</td>
<td>216 (69)</td>
<td>96 (31)</td>
</tr>
<tr>
<td>&gt;200–400 mg/d</td>
<td>714 (71)</td>
<td>291 (29)</td>
</tr>
<tr>
<td>&gt;400 mg/d</td>
<td>174 (71)</td>
<td>71 (29)</td>
</tr>
<tr>
<td>P for trend</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smokers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100 mg/d</td>
<td>23 (51)</td>
<td>22 (49)</td>
</tr>
<tr>
<td>100–200 mg/d</td>
<td>45 (58)</td>
<td>33 (42)</td>
</tr>
<tr>
<td>&gt;200–400 mg/d</td>
<td>348 (69)</td>
<td>155 (31)</td>
</tr>
<tr>
<td>&gt;400 mg/d</td>
<td>252 (74)</td>
<td>90 (26)</td>
</tr>
<tr>
<td>P for trend</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results were determined by unconditional logistic regression. P = 0.07 for caffeine × smoking interaction was determined by the −2log ratio test.

#### FIGURE 1.
Frequency of nonsmokers and current smokers consuming <100 mg caffeine/d by cytochrome P4501A2 (CYP1A2) genotype [P = 0.62 for nonsmokers (11.1% compared with 12.0%) and P = 0.32 for current smokers (4.0% compared with 5.2%)] and adenosine A2A receptor (ADORA2A) genotype [P = 0.011 for nonsmokers (10.3% compared with 14.6%) and P = 0.008 for current smokers (3.4% compared with 7.3%)]. Results are from Pearson’s chi-square test with 1 df. The ADORA2A × smoking interaction was not significant for either genotype.
caffeine intake. This observation provides a biological basis for caffeine consumption behavior and suggests that persons with this genotype may be less vulnerable to caffeine dependence.

Our results are consistent with evidence showing the important role that behavioral responses to caffeine play in habitual caffeine consumption (1, 6, 7). However, the role of other genetic or environmental factors affecting caffeine consumption cannot be excluded. For example, genetic differences in taste were shown to affect how persons rate the bitter taste of caffeine, which may in turn affect their preference for caffeinated beverages (33). We excluded persons with a history of hypertension, but some persons may avoid caffeinated beverages because of other perceived adverse health effects. Finally, the social context in which caffeinated beverages are consumed could also contribute to habitual caffeine consumption. These factors, however, would have attenuated the effect of ADORA2A genotype on caffeine consumption.

In summary, genetic variation in the A2A receptor, the main target of caffeine action in the CNS, is associated with caffeine consumption in a free-living population. The association between ADORA2A genotype and caffeine consumption suggests that this genetic variant might be a confounder in observational studies that relate caffeine intake to certain health outcomes. Variation in the adenosinergic system also may be an important factor in studies of a genetic predisposition to caffeine dependence, a subject of ongoing debate (1, 5).

We thank Xinia Siles (project director at the Centroamericano de Población, Universidad de Costa Rica) for directing the data collection and Ana Baylin (Department of Nutrition, Harvard University School of Public Health) for data monitoring and management throughout the study.

The authors’ responsibilities were as follows—MCC: completed the genotyping, performed statistical analysis, and prepared the first draft of the manuscript; AE-S and HC: obtained funding and provided supervision; and all authors: contributed to data interpretation and critically reviewed the manuscript. None of the authors had a personal or financial conflict of interest.

REFERENCES

Bone mass and soy isoflavones in socially housed, premenopausal macaques1–3

Cynthia J Lees, Jay R Kaplan, Haiying Chen, Christopher P Jerome, Thomas C Register, and Adrian A Franke

ABSTRACT
Background: Soy consumption is associated with a lower incidence of hip fracture in Asian than in Western women, an effect often attributed to estrogen-like compounds (isoflavones) in soy. It is not known whether premenopausal soy exposure initiated in adulthood can increase bone mass and thereby reduce fracture risk.

Objective: We aimed to determine whether a high-isoflavone soy diet influences bone mass in soy-naïve, premenopausal cynomolgus monkeys (Macaca fascicularis).

Design: Ninety-four skeletally mature females were randomly assigned to consume diets whose protein content came from either high-isoflavone soy or casein and lactalbumin. Animals were socially housed. Bone mass and circulating isoflavone concentrations were measured at baseline and 19 and 31 mo after the start of treatment; bone biomarkers were measured at baseline and 31 mo.

Results: There were no significant differences at any timepoint in whole-body bone mineral content between casein-fed (112.5 ± 2.1, 119.2 ± 1.9, and 120.7 ± 2.1 g) and soy-fed (117.2 ± 2.1, 122.4 ± 2.0, and 125.4 ± 2.3 g; P = 0.12) monkeys. Similar results were seen for spinal bone mineral density (casein-fed: 0.46 ± 0.01, 0.50 ± 0.01, and 0.52 ± 0.01 g/cm²; soy-fed: 0.47 ± 0.01, 0.51 ± 0.01, and 0.52 ± 0.01 g/cm²; P = 0.30) and bone biomarker measurements—bone-specific alkaline phosphatase (soy-fed: 82.3 ± 4.1 and 63.2 ± 3.4 ng/mL; casein-fed: 94.1 ± 4.5 and 61.7 ± 4.3 ng/mL; P = 0.22) and C-terminal crosslink of type 1 collagen (soy-fed: 0.944 ± 0.06 and 0.89 ± 0.08 nmol/L; casein-fed: 0.97 ± 0.07 and 0.78 ± 0.06 nmol/L; P = 0.20).

Conclusion: A soy diet high in isoflavones does not significantly affect bone characteristics in initially soy-naïve premenopausal monkeys.


KEY WORDS Premenopausal monkeys, Macaca fascicularis, soy protein, isoflavones, bone mass, bone biomarkers

INTRODUCTION
Fully half of all postmenopausal women will experience an osteoporosis-related fracture and associated morbidity (1, 2). Current methods for preventing osteoporosis and associated fractures include hormone replacement therapy (HRT), bisphosphonates, and selective estrogen receptor modulators (SERMs). However, these postmenopausal treatment modalities all have limitations or potential adverse side effects. Premenopausal interventions that promote maximal peak bone mass could increase resistance to future fracture and thereby reduce the need for postmenopausal treatment (3, 4).

Estrogen plays a critical role in the premenopausal acquisition and maintenance of peak bone mass (5–8). This observation, in turn, has led to speculation that the estrogen-like compounds (isoflavones) contained in soy protein may reduce resorption and enhance peak bone mass acquisition (9, 10). A series of observational studies evaluating premenopausal Asian women suggested that habitual consumption of soy protein or isoflavone is associated with the preservation of bone mass (11–13). However, at least one observational study of premenopausal Chinese women did not find such an association (14), and a randomized trial showed that isoflavone exposure had no effect on bone density among relatively young (21–25 y) women, most of whom were white (15).

The geographic epidemiology relating soy consumption to health benefits is based in part on Asian populations with extensive lifetime exposure to soy that begins in utero, that is much lower (but not zero) during breastfeeding, and that is elevated again when solid foods are consumed (16, 17). In contrast, typical Western persons, if raised on soy formula, experience high isoflavone exposure only during infancy; if not raised on soy formula, they are generally soy-naïve until adulthood (18). Soy supplementation to adults with such different histories of soy exposure could potentially have divergent effects on estrogen-sensitive tissues such as bone (18, 19).

To further explore the effect of premenopausal soy exposure on bone mass, we evaluated the responses of skeletally mature cynomolgus monkeys (Macaca fascicularis) to diets deriving most of their protein from either high isoflavone, isolated soy protein, or a casein-lactalbumin mixture. This animal model, which closely resembles women in menstrual cyclicity and with an ovarian hormone profile that closely resembles that of women in reproductive physiology, has been used extensively to assess the effects of dietary, hormonal, and behavioral manipulations on skeletal indices and cardiovascular disease risk (20). The primary objectives of the current study were to determine whether

1 From the Wake Forest University School of Medicine, Winston-Salem, NC (CJL, JRK, HC, CPJ, and TCR), and the Cancer Research Center of Hawaii, Honolulu, HI (AAF).

2 Supported by grants no. HL45666 and HL079421 from the National Heart, Lung, and Blood Institute (to JRK) and grant no. CA71789 from the National Cancer Institute (to AAF).

3 Reprints not available. Address correspondence to CJ Lees, Department of Pathology, Section on Comparative Medicine, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157-040. E-mail: clee@wfubmc.edu.

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soy consumption was associated with alterations in whole-body bone mineral content (WBBMC), spinal (lumbar vertebrae 2–4) bone mineral density (BMD), or serum biomarkers of bone turnover in premenopausal subjects.

MATERIALS AND METHODS

Animals

The subjects were 94 female cynomolgus monkeys imported from Indonesia (Institute Pernanian, Bogor, Indonesia). Before shipment, all animals were radiographed. Only individuals exhibiting evidence of complete epiphyseal closure at the distal radius and ulna and the proximal tibia were used in the study. This stage of development generally occurs by 9 or 10 y of age in cynomolgus monkeys, at which time individuals are approximately equivalent to 25-y-old women (21, 22). Peak bone mass attainment lags behind epiphyseal closure, although its trajectory is generally set during the period between puberty and epiphyseal closure (23). Additional radiographs of the spine were examined for any predisposing disease or injury that could potentially confound any spine density measurements. Finally, animals had no known prior exposure to soy and were thus comparable to fully mature, premenopausal women that are developmentally naïve to isoflavones.

All animal manipulations were performed according to the guidelines of state and federal laws, the US Department of Health and Human Services, and the Animal Care and Use Committee, Wake Forest University School of Medicine. Wake Forest University is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

Housing, diets, and assessments

After their arrival at our facility, the animals were placed in single cages for a federally mandated 30-d quarantine period. On release from quarantine, the animals were placed in 16 social groups of 5 or 6 animals each; the groups were approximately equivalent in body weight gradient. For the next 8 mo, animals consumed an isoflavone-free diet with 19% of calories from protein, 44% of calories from fat, 37% of calories from carbohydrate, and 0.20 mg cholesterol/kg. This diet was designed to model the high-fat, high-cholesterol diet often consumed by Americans and thought to contribute to both obesity and cardiovascular disease (24). Three months after the initiation of this experimental diet and the final bone density determinations. These animals died from a variety of unrelated causes that could not be attributed to the experimental manipulation, including arterial disease, congestive heart failure, meningitis, acute enteritis, and oligoendroglioma.

Bone densitometry and body weight

WBBMC (g) and spinal BMD (g/cm²) were measured at baseline (≈6 mo before starting the experimental diet) and 19 and 31 mo after the start of the diet by using a dual-energy X-ray absorptiometry machine (DXA) XR-46; Norland, Fort Atkinson, WI) with DXA software (version 3.9.6b; Norland). The monkeys were sedated with ketamine (10 mg/kg) and then given isoflurane. Measurements were made of the whole body and the lumbar spine (vertebrae 2–4) by using techniques described previously (25, 26). The CVs were 1.7% for WBBMC and 2.1% for spinal BMD.

Animals were sedated every 4–6 wk to determine body weight (model #815 scale; Chatillon, Largo, FL). The amount of diet fed was adjusted to changes in body weight to ensure that the animals continued to receive 120 kcal/kg (+ 10% wastage).

Isoflavone concentrations

Serum samples were collected on 3 occasions from each monkey for the measurement of circulating isoflavone concentrations. These collections occurred at baseline and 19 and 31 mo after the initiation of dietary treatment. Animals were fed in the morning and then sedated 4 h after feeding for blood collection. Blood was immediately processed, frozen, and protected from light until analysis. Serum isoflavones were analyzed by using liquid chromatography photo-diode array mass spectrometry that was slightly modified from a previously established method (27) to include equol in the panel of isoflavonoids (genistein, dihydrogenistein, daidzein, dihydrodaidzin, glycitein, and O-desmethylangolensin) and isotopically labeled internal standards (28, 29). Detection limits were previously found to be 1–15
nmol/L, depending on the analyte, and interassay CVs were 8–22% at concentrations <20 nmol/L, 7–14% at concentrations of 20 to 100 nmol/L, and 3–12% at concentrations >100 nmol/L.

Serum biomarkers of bone metabolism

Baseline (6 mo before experimental diets) and 31-mo serum samples were collected and stored frozen at −70 °C until they could be assayed for serum bone biomarkers [bone-specific alkaline phosphatase (BALP) and C-terminal crosslink of Type I collagen (CTX)]. Serum BALP activity was measured by using an immunocapture assay (Metra BAP; Quidel Corp, San Diego, CA). Serum CTX was measured by using a commercially available enzyme-linked immunosorbent assay (Serum Crosslapps; Nordic Biosciences Diagnostics, Helev, Denmark) that was specific for the amino sequence EKAHD-β-GGR derived from the C-terminal telopeptide region of type I collagen. Intraassay and interassay CVs for serum BALP and CTX were <10%.

Behavioral observations and dominance determinations

Because individual differences in social status were previously observed to influence bone density and reproductive hormones, status was evaluated in relation to soy treatment to determine whether there were any confounding effects (20). The social status of each animal in relation to others in her social group was based on data collected during weekly, 30-min observations beginning after social group formation and before the initiation of dietary intervention. Dominance and subordination were determined by the outcomes of fights, which are highly asymmetric, and they yield clear winners and losers as judged by specific facial expressions, postures, and vocalizations (30, 31). The females included in this study had completed long bone growth. Nonetheless, all monkeys achieved an increase in bone mass across time (WBBMC from 114.86±1.5 to 123.07±1.6 g; P < 0.001; spinal BMD from 0.47±0.00 to 0.52±0.01 g/cm²; P < 0.001), a process accompanied by a significant, 20% increase in body weight that coincided with consumption of the experimental (high-fat) diets (from 2.92±0.04 kg to 3.51±0.07 kg, P < 0.001). The increases in bone mass and body weight occurred irrespective of dietary condition.

The effects of dietary treatment (soy or CL diet) on WBBMC and spinal BMD were compared longitudinally with the main effects of diet and time and the diet × time interaction after adjustment for the corresponding body weight at each time-point. For the bone biomarkers BALP and CTX, the 24-mo follow-up measurements were modeled with the main effect of diet treatment and covariates including baseline measurements. Type 3 F tests were used to test the significance of the variables in the model, and all analyses were performed by using SAS software (version 9.1; SAS Institute, Cary, NC).

RESULTS

Data from all subjects that started the study (n = 94) contributed to the analyses. Forty-eight animals were assigned to the CL condition and the remaining 46 to the soy condition.

Consumption of the soy diet resulted in high concentrations of plasma daidzein, genistein, and equol (Table 1). Trace amounts of these compounds were detected in most monkeys at baseline and in the CL condition monkeys during the treatment period. The females included in this study had completed long bone growth. Nonetheless, all monkeys achieved an increase in bone mass across time (WBBMC from 114.86±1.5 to 123.07±1.6 g; P < 0.001; spinal BMD from 0.47±0.00 to 0.52±0.01 g/cm²; P < 0.001), a process accompanied by a significant, 20% increase in body weight that coincided with consumption of the experimental (high-fat) diets (from 2.92±0.04 kg to 3.51±0.07 kg, P < 0.001). The increases in bone mass and body weight occurred irrespective of dietary condition.

The effects of dietary treatment (soy or CL diet) on WBBMC and spinal BMD across time are shown in Figure 1 and Figure 2. Although the trend lines for both outcomes appeared to diverge over the course of the study, the treatment effects did not reach conventional significance as either a main effect (P = 0.12 for WBBMC and 0.29 for spinal BMD) or in interaction with time (P = 0.37 for WBBMC and 0.44 for spinal BMD). Nor did dietary treatment affect body weight, either as a main effect or in interaction with time (P > 0.20 for both; Figure 3).

### TABLE 1

Plasma concentrations of genistein, daidzein, and equol in monkeys fed casein lactalbumin or high-isoflavone soy protein

<table>
<thead>
<tr>
<th></th>
<th>Casein lactalbumin (n = 48)</th>
<th>High-isoflavone soy protein (n = 46)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline 19 mo 31 mo</td>
<td>Baseline 19 mo 31 mo</td>
</tr>
<tr>
<td>Genistein</td>
<td>0.8±0.5 0±0 4.6±2.1</td>
<td>0±0 210.8±25.0 123.3±14.6</td>
</tr>
<tr>
<td>Daidzein</td>
<td>2.4±1.2 0.6±0.4 9.8±2.6</td>
<td>2.8±1.1 293.6±33.6 128.8±15.9</td>
</tr>
<tr>
<td>Equol</td>
<td>6.5±0.5 0±0 7.6±3.1</td>
<td>0.8±0.8 686.3±63.0 546.1±45.5</td>
</tr>
</tbody>
</table>

1 All values are x±SE.
At the end of the treatment period, the bone biomarkers BALP and CTX did not differ significantly between dietary treatment groups ($P > 0.20$; Table 2). However, individuals with greater bone mass had lower BALP concentrations, as indicated by the significant negative correlation between WBBMC and BALP ($P < 0.05$). Spinal BMD was also negatively associated with BALP, although the correlation coefficient was not significant ($P < 0.10$). Serum values of CTX were not significantly associated with indexes of bone mass ($P > 0.20$ for all).

**DISCUSSION**

The purpose of the present study was to determine whether consumption of a diet high in soy protein and isoflavones alters bone mass in skeletally mature, soy-naïve premenopausal monkeys. These data indicate that consumption of a soy protein-based diet containing isoflavones in an amount that approximates 129 mg/d for a woman resulted in substantially elevated plasma isoflavone concentrations. However, whereas WBBMC and spinal BMD increased in all individuals over time, there were no significant differences related to dietary treatment. Among other results, the soy diet had no significant effect on BALP, although, for all monkeys, that biomarker was significantly related to bone mass. All animals experienced a significant increase in body weight during the experiment, an effect that was independent of dietary treatment.

Asian women, whose diet typically contains soy, are postmenopausally at lower risk of hip fracture than are white women (33–35). This observation has led to the suggestion that lifelong consumption of soy isoflavones exerts a protective effect in postmenopausal women, either by increasing premenopausal peak bone mass or by decreasing the rate of postmenopausal bone loss. To date, studies done in premenopausal women or nonovariectomized animal models suggest that soy exposure has no consistent beneficial effect on bone in estrogen-normal individuals. For example, in one prospective trial, isoflavone supplementation for 1 y had no significant effect on bone mass among normally

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**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>Casein lactalbumin ($n = 48$)</th>
<th>High-isoflavone soy protein ($n = 46$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>31 mo</td>
</tr>
<tr>
<td>BALP (ng/mL)</td>
<td>$94.1 \pm 4.5$</td>
<td>$61.7 \pm 4.3$</td>
</tr>
<tr>
<td>CTX (nmol/L)</td>
<td>$0.97 \pm 0.07$</td>
<td>$0.78 \pm 0.06$</td>
</tr>
</tbody>
</table>

$^1$ All values are $\bar{x} \pm$ SEM. Linear mixed-effects modeling was used for statistical analyses. There were no significant differences between dietary conditions at either time ($P > 0.2$ for both measurements).
cycling women in their 20’s (15). This study specifically evaluated the effect of isoflavones, because both the control (n = 13) and treatment (n = 15) groups received the same amount of alcohol-extracted (isoflavone-deficient) soy protein, whereas the treatment group received a supplement containing 90 mg isoflavones/d. In turn, this outcome is consistent with a premenopausal cohort study indicating that differences in isoflavone consumption are not associated with variation in bone mass (14). Similarly, studies using reproductively intact rats or mice usually do not find significant increases in bone mass after soy or isoflavone supplementation (36, 37).

The current results in nonhuman primates are consistent with the foregoing data from humans and rats, and they support the conclusion that an isoflavone-rich soy diet does not improve bone mass indexes when fed chronically to fully mature, premenopausal individuals. Notably, there is no indication of a negative effect on bone, as might be expected had soy isoflavones interfered substantially with endogenous estrogenic activity. As reported elsewhere, consumption of the soy-based diet had no significant effects on menstrual cyclicity or the concentrations of reproductive hormones (24). Inasmuch as the present study focused on monkeys that had minimal prior exposure to soy and isoflavones, these observations are relevant to the likely effect of soy supplementation on typical premenopausal American women—a group of persons who generally consume little soy during the juvenile or adolescent years and who were not exposed in utero.

It has been suggested that the absence of effects in premenopausal subjects may relate to the inability of isoflavones to exert estrogenic effects when adequate endogenous estrogen is available (19). This hypothesis implies that soy isoflavones may exert an estrogen agonist–like effect in estrogen-deficient persons. In fact, ≥3 randomized trials in relatively young, postmenopausal women have indicated that soy isoflavones exert bone-sparing effects (38–40). In contrast, the Utrecht trial—a study in which women were an average of 18 y past menopause—did not find significant increases in bone mass after soy or isoflavone supplementation (36, 37).

Although radiographic evidence indicated that all females in the present experiment had completed long bone growth, all monkeys nonetheless experienced an increase in bone mass across the study. Similarly, women generally accumulate bone mass into their 30s, even though bone lengthening stops much earlier (23). In the current study, the outcome may also have been influenced by an increase in weight as the animals began consuming experimental diets that were calorically dense as well as high in fat. Another factor affecting bone mass may have been dietary calcium, because these monkeys received calcium at a dose equivalent to that received by a well-supplemented woman (ie, 1300 mg/d).

In summary, chronic consumption of a high-isoflavone soy protein diet did not significantly affect bone mass in skeletally mature, premenopausal monkeys. A similar null finding may be expected among women in industrialized countries, who are largely soy-naïve until adulthood and who are living in a calorically adequate environment.

The authors’ responsibilities were as follows—CJL (primary author): the collection and interpretation of the dependent measures; JRK (principal investigator): the design and overall conduct of the study; HC: the interpretation of results; CPJ: participation in the conception and design of the study; TCR: bone biomarker data; and AAF: the isoflavone analyses. None of the authors had a personal or financial conflict of interest.

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Bone mineral density and bone markers in patients with a recent low-energy fracture: effect of 1 y of treatment with calcium and vitamin D<sup>1–3</sup>

Mette F Hitz, Jens-Erik B Jensen, and Peter C Eskildsen

**ABSTRACT**

**Background:** Low-energy fractures of the hip, forearm, shoulder, and spine are known consequences of osteoporosis.

**Objective:** We evaluated the effect of 1 y of treatment with calcium and vitamin D on bone mineral density (BMD) and bone markers in patients with a recent low-energy fracture.

**Design:** In a double-blinded design, patients with fracture of the hip (lower-extremity fracture, or LEF) or upper extremity (UEF) were randomly assigned to receive 3000 mg calcium carbonate plus 1400 IU cholecalciferol or placebo (200 IU cholecalciferol). BMD of the hip (HBMD) and lumbar spine (LBMD) were evaluated by dual-energy X-ray absorptiometry, and physical performance was assessed by the timed Up & Go test. Serum concentrations of 25-hydroxycholecalciferol, parathyroid hormone (PTH), telepeptide of type I collagen (ICTP), osteocalcin, and N-terminal propeptide of collagen type I were measured.

**Results:** A total of 122 patients were included (84% women; mean ± SD age: 70 ± 11 y); 68% completed the study. In an intention-to-treat analysis, LBMD increased in the intervention group and decreased in the placebo group, and the difference between the groups was significant after 12 mo: 0.931 ± 0.211 compared with 0.848 ± 0.194 (P < 0.05). No significant change was shown for HBMD. The effect of treatment was more pronounced in patients aged <70 y. The intervention decreased bone turnover. PTH was significantly lower in the intervention group (P < 0.01) for the LEF patients. ICTP and change in LBMD were significantly related to physical performance.

**Conclusions:** A 1-y intervention with calcium and vitamin D reduced bone turnover, significantly increased BMD in patients younger than 70 y, and decreased bone loss in older patients. The effect of treatment was related to physical performance. *Am J Clin Nutr* 2007;86:251–9.

**KEY WORDS** Bone markers, bone mineral density, calcium, immobility, low-energy fracture, hip fracture, shoulder fracture, forearm fracture, vitamin D

**INTRODUCTION**

Low-energy fractures of the hip, forearm, shoulder, and spine are known consequences of osteoporosis (1–3). Hip fractures occur mainly in older individuals at risk of being deficient in calcium and vitamin D (4). Calcium and vitamin D deficiencies result in the elevation of parathyroid hormone (PTH), increased bone remodeling, and skeletal loss of calcium (5). Bone turnover and serum concentrations of biochemical bone markers increase in women after menopause. A disequilibrium arises when the amount of bone resorption is higher than the amount of bone formed, and net loss of bone is seen (6).

Correction of calcium and vitamin D deficiencies has been investigated in clinical trials, and positive effects on bone remodeling, bone mineral density (BMD), rate of falling, and fracture incidences have been shown, but studies evaluating the effects of intervention in patients with a recent low-energy fracture are sparse (7–10). A fracture episode, the subsequent surgical trauma, and immobilization all affect the serum concentration of bone markers and the formation of new bone (11). To be able to use the measurement of biochemical markers of bone remodeling in the evaluation of bone turnover in patients with recent fractures, more studies investigating the concentration of bone turnover markers in the post-fracture period and the effect of intervention with calcium and vitamin D are needed. Bone turnover increases as soon as 24 h after a patient is immobilized, with a subsequent release of calcium and suppression of PTH. Immobilization after a fracture also influences bone turnover, but whether supplementation with calcium and vitamin D can reduce this turnover is unknown.

The aim of the present study was to investigate the effect of treatment with 3000 mg calcium carbonate plus 1400 IU cholecalciferol in a group of patients with a recent low-energy fracture of the hip, distal forearm, or proximal humerus. The effect of treatment was evaluated by the measurement of BMD at the hip and lumbar spine region and by the measurement of biochemical markers of bone remodeling.

**SUBJECTS AND METHODS**

**Patients**

Women and men admitted to Roskilde Amt University Hospital in Koge with a relevant low-energy fracture and aged

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>50 y were included in the study. All women were postmenopausal (>2 y since last menstruation).

Patients with dementia or in such a state that their ability to sign an informed consent form was affected were excluded from the study. Patients with a history of cancer except superficial skin cancers or with excessive alcohol abuse were also excluded. Excessive alcohol abuse was identified by history of abuse and by hospital records and was defined as a use of alcohol exceeding the recommendations of the Danish Health Board of no more than 21 units of alcohol per week for men and no more than 14 units of alcohol per week for women.

Patients using hormone replacement therapy, taking medication for the treatment of osteoporosis, having an intake of cholecalciferol >800 IU, or using 1,25-dihydroxycholecalciferol were excluded, as were patients with serum creatinine concentrations >130 μmol/L or serum alanine aminotransferase >2.5 times the upper limit.

During the inclusion period (18 mo), 277 patients with a low-energy hip fracture were screened for participation, and 117 hip fracture patients were eligible according to the inclusion criteria. Of these, 23% did not wish to participate; the most frequently stated reasons for not wanting to participate in the trial were feeling too old, sensation of poor health, and a wish to commence treatment with anti-osteoporotic medication.

A total of 141 patients with a fracture of the distal forearm and 46 patients with a fracture of the proximal humerus were invited to participate in the study; 34% and 41% of these were included in the study. The number of included individuals was 122 patients: 55 hip-fracture patients, 48 forearm fracture patients, and 19 proximal humerus fracture patients. The group consisted of 101 women and 21 men.

**Design and methods**

The hip-fracture patients were included consecutively over a period of 18 mo and were informed about the study while admitted to the hospital. Differences in the health status of the fracture patients postoperatively resulted in variation in the time after fracture to inclusion into the study. Variation in time to inclusion had to be accepted, even though time since fracture has an important influence on the concentrations of bone markers.

The patients with fracture of the upper extremity were informed about the study through the mail and were subsequently invited to a meeting if they met the inclusion criteria. The upper-extremity fracture patients were recruited over a period of 12 mo.

The study was prospective and double-blinded, and patients were randomly assigned in blocks to one of the following regimens (total daily dose): 1) 3000 mg calcium carbonate (corresponding to a dose of 1200 mg elemental calcium) + 1200 IU cholecalciferol and 200 IU cholecalciferol given as a multivitamin tablet (a total of 1400 IU corresponding to 35 μg cholecalciferol), or 2) placebo tablets + 200 IU cholecalciferol given as a multivitamin tablet (5 μg cholecalciferol). The tablets looked identical and had the same flavor. The regimens were taken as one tablet with a meal 3 times daily plus a multivitamin tablet. Pharma Vinci A/S, Frederiksvaerk, Denmark, performed the blinding and coding. Compliance was recorded by counting the tablets at all visits.

History of self-reported use of vitamin tablets and vitamin D supplements (<800 IU) was recorded, as were smoking habits and alcohol use. Smoking habits were recorded by using a frequency questionnaire, recording the number of pack-years (20 cigarettes per day) ever smoked. Alcohol use was recorded as a mean of consumed units of alcohol per week. Calcium intake was recorded by using a food-frequency questionnaire and was calculated from the intake of dairy products with the addition of 300 mg daily for all other food products. Body mass index (BMI) and age at menopause were recorded as well.

**Bone mineral density**

BMD was measured at the hip region of the nonfractured hip for the hip fracture patients and of the left hip if possible for patients with upper-extremity fractures. Of the hip fracture patients, 8 did not undergo a dual-energy X-ray absorptiometry scan of the hip because of the presence of osteosynthetic material from a previous fracture episode or total hip replacement caused by osteoarthritis.

As a result of recent surgical repair, many of the patients with a fracture of the hip were unable to elevate their legs during the lumbar spine scanning procedure; as a result, all hip fracture patients were scanned without their legs elevated. Double scans were obtained in a group of 23 healthy women both with and without leg elevation. Investigation for any systematic difference was evaluated from a Bland-Altman plot; the mean difference between the 2 scanning procedures was 0% (SD: 1.78), which resulted in an overestimation of the BMD value for the procedure in which the patients’ legs were not elevated.

For the patients with upper-extremity fractures, the lumbar scan was performed with the patients’ legs elevated. For all patients, no effort was made to scan the side of dominance, because side of dominance has been shown to affect only BMD of the upper extremities (1)2.

Dual-energy X-ray absorptiometry with a Hologic QDR-2000 (Hologic Inc, Waltham, MA) was used to measure BMD at the hip and lumbar spine. Hologic System Software version 7.10 was used for subsequent analyses. The software-provided reference database was used. This reference is used as a standard in Denmark (13). Variation between identical double scans (CV for scanner) was 1.3% for the lumbar spine region and 1.5% for the hip region on the basis of 20 duplicate measurements.

Physical performance was evaluated at inclusion and after 12 mo. The timed Up & Go test was used to evaluate physical performance (14).

**Ethical approval**

The study was designed according to the Helsinki II Declaration and was approved by the local scientific ethical committee. Informed consent was obtained from all participants before inclusion.

**Sample collection and analysis**

Control visits were performed 1, 3, and 12 mo after inclusion. Automated chemical analysis was used for analysis of sodium, potassium, creatinine, alkaline phosphatase, alanine aminotransferase, calcium, phosphate, and thyrotropin in serum (T; reference value: 0.82-5 nmol/L) and free thyroxine (FT; reference value: 7.0–22.0 pmol/L). To minimize the effect of circadian rhythm on markers of bone remodeling, blood tests were obtained before noon with a time window of 3 h duration. All sample analyses were performed blinded.
Serum concentrations of intact N-terminal propeptide of type I collagen (PINP) were measured by using the UniQ PINP radioimmunoassay (Orion Diagnostica, Espoo, Finland). The intraassay CVs were 4.8% (for a concentration of 39 µg/L) and 8.0% (for a concentration of 90 µg/L). The interassay CVs were 3.1% (for a concentration of 27 µg/L) and 4.6% (for a concentration of 78 µg/L).

Serum osteocalcin concentrations were measured with the N-MID Osteocalcin One Step ELISA Kit (Nordic Bioscience Diagnostics A/S, Herlev, Denmark). The intraassay CVs were 3.4% (for a concentration of 7.0 ng/mL) and 2.4% (for a concentration of 43.2 ng/mL). The interassay CVs were 3.6% (for a concentration of 6.8 ng/mL) and 6.4% (for a concentration of 30.5 ng/mL).

Serum concentrations of C-terminal telopeptide of type I collagen (ICTP) were measured with the UniQ ICTP radioimmunoassay (Orion Diagnostica, Finland). The intraassay CVs were 3.5% (for a concentration of 3.7 ng/mL) and 9.4% (for a concentration of 24.5 ng/mL). The interassay CVs were 5.6% (for a concentration of 5.5 ng/mL) and 9.0% (for a concentration of 21.3 ng/mL).

Vitamin D status [25-hydroxyvitamin D2 [25(OH)D2] and 25(OH)D3] was measured in serum by using the Gamma-B-Hydroxy Vitamin D radioimmunoassay from ImmunoDiagnostics System, Boldon, UK. The intraassay precision was 5.0% for a mean of 58.4 nmol/L and the interassay precision was 8.1% for a mean of 56.7 nmol/L (for vitamin D, concentrations > 50 nmol/L are considered normal).

Serum intact PTH was measured by using an immunometric assay with CVs of 15% (for a concentration of 2.4 pmol), 10% (for a concentration of 6.3 pmol), and 12% (for a concentration of 22.8 pmol) (Immulite 2000; Diagnostic Products Corporation, Los Angeles, CA). All samples were stored at −80 °C during the study, and samples from each patient were analyzed by using the same assay to minimize interassay variations.

Safety
Serum ionized calcium concentrations were measured at baseline and after 1, 3, and 12 mo of intervention. Furthermore, additional serum calcium measurements were performed if hypercalcemia was suspected or if a rise in serum calcium was observed. No cases of hypercalcemia were recorded during the study.

Statistics
The normally distributed data were analyzed by using parametric statistics. For the skewed data, a normal distribution was obtained by logarithmic transformation, and statistical analysis was performed on the log-transformed data. Independent-sample t tests were used to compare means with the use of SPSS 11.5 for WINDOWS (SPSS Inc, Chicago, IL). To analyze the longitudinal data (biochemical parameters), a mixed general linear model was used to account for the stronger association between variables in the same individual over time using SAS 9.1 (SAS Institute Inc, Cary, NC). Only data from patients completing the study were included in this analysis.

To evaluate the effect of treatment for 1 y with calcium and vitamin D on BMD, we included both an intention-to-treat analysis and an evaluation of the treatment effect for the group of participants who completed the study to investigate the biological effect when receiving the intervention in a per protocol analysis. Pearson’s correlation coefficients or general linear model was used to analyze relations between variables.

Power calculations

Prestudy power calculations
The intervention was expected to result in an increase in BMD for the active intervention group of ≥8%. The level of significance was set to 5% (SD = 0.08) and the power of the study to 80%. Significant results were expected after the inclusion of 60 patients.

For biochemical markers of bone turnover, the intervention was expected to result in a change in biochemical markers of 1 SD. The power of the study was again set to 80% and the level of significance to 5%. With this design, significant results would be obtained after the inclusion of 16 patients in each group.

Poststudy power calculations
These were performed for those variables for which a significant effect of treatment could not be shown. This was done to evaluate whether a type II error was present.

The actual power of the study in an intention-to-treat analysis was calculated to be 57.7% for the BMD of the hip. In the per-protocol analysis, the actual power of the study was 73.2% for BMD of the lumbar spine and 29.9% for BMD of the hip.

RESULTS
Of the 122 included patients, 79 (68%) completed the study: 53% of the patients with a hip fracture, 74% of the patients with a fracture of the proximal humerus, and 75% of the patients with a fracture of the distal forearm. The reasons for dropping out were as follows: 27% of the patients wanted to be excluded, 1% were excluded because of compliance problems, and 4% of the patients died.

Compliance in adherence to the treatment regimens in taking the tablets every day was 95% for the patients who completed the study (95 of 100 tablets were consumed). We compared the baseline values of those who completed the study with those who did not and found no significant differences except in age at menopause (P < 0.001), for which those who did not complete the study were significantly younger at menopause.

Baseline data
The baseline characteristics of all fracture patients are shown in Table 1. No significant differences were found between patients randomly assigned to the active treatment group and those assigned to the placebo group except in menopausal age, which was significantly lower in the placebo group (P < 0.05). In a regression analysis, however, menopausal age had no significant effect on BMD. There was a strong tendency toward a higher BMD at the spine region for patients randomly assigned to the active treatment, though the difference was not significant (P = 0.06).

Compared with the upper-extremity fracture patients, the hip fracture patients were older (P < 0.001), had lower BMIs (P < 0.001), tended toward a lower calcium intake (P < 0.09), had a lower intake of alcohol (P < 0.001), and had a lower hip BMD (P < 0.001). The hip fracture group was also characterized as having low vitamin D concentrations without elevation of PTH. The hip fracture patients took longer to perform the timed Up &
related with time since fracture episode and time since surgical procedure, and the upper-extremity fracture group was included 26.8 d after fracture; the difference was significant (P < 0.01). Changes in serum 25(OH)D were significant for both intervention groups after 1, 3, and 12 mo (P < 0.01; Figure 1B).

**Effect of treatment**

The hip fracture group was included 13.6 ± 11.0 d after fracture, and the upper-extremity fracture group was included 26.8 ± 15.5 d after fracture; the difference was significant (P < 0.01). Because serum concentrations of osteocalcin were positively related with time since fracture episode and time since surgical trauma (r = 0.242, P < 0.05), we chose to evaluate the effect of treatment with calcium and vitamin D on bone markers for the 2 fracture groups separately.

**Vitamin D**

For the hip fracture group, the intervention increased the vitamin D concentration from 33 ± 17 to 82 ± 19 nmol/L, whereas the placebo group increased only from 40 ± 17 to 53 ± 16 nmol/L. The changes in serum 25(OH)D were significant for both intervention groups after 1 mo. Serum concentrations of 25(OH)D were significantly higher in the active intervention groups after 1, 3, and 12 mo (P < 0.001). Changes in serum 25(OH)D are shown in Figure 1A and Figure 2A.

For the upper-extremity fracture group, the intervention increased the vitamin D concentration increased from 63 ± 18 to 90 ± 24 nmol/L (P < 0.001) for the intervention group and from 69 ± 34 to 77 ± 31 nmol/L (P < 0.01) for the placebo group. The increase was significantly higher in the active intervention group than in the placebo group (Figure 2).

**Parathyroid hormone**

**Hip fracture group.** Serum PTH was significantly lower in the active intervention group at 1, 3, and 12 mo than in the placebo group, but the change compared with baseline was not significant for the active intervention group. PTH increased in the placebo group, and the difference between intervention groups was significant after 12 mo (P < 0.001; Figure 1B).

**Upper-extremity fracture group.** PTH was significantly lower in the active intervention group at 1 and 3 mo than in the placebo group, and the change compared with baseline was significant after 1, 3, and 12 mo (P < 0.001) for the active intervention group but not for the placebo group (Figure 2B).

**Bone markers**

**Hip fracture group.** To account for differences present already at baseline, we included baseline as a covariate in a repeated-measures analysis of variance (mixed model) and found the difference between treatments to remain significant (P < 0.05).

PINP increased significantly in both groups after 1 mo (P < 0.01) and declined at 3 and 12 mo. PINP was lower in the intervention group at all visits than in the placebo group (P < 0.05) and had reached a lower level after 12 mo for both intervention groups compared with baseline (P < 0.001; Figure 1C).

Osteocalcin increased from baseline to 12 mo in both groups. Osteocalcin concentrations were significantly higher in the placebo group than in the active intervention group after 1, 3, and 12 mo (P < 0.05; Figure 1D).

ICTP increased for both intervention groups after 1 mo but not significantly. Then ICTP declined and after 12 mo was below the baseline concentration for both intervention groups (P < 0.05).

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**TABLE 1**

Baseline data for all patients completing the study by fracture group

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hip fracture group</th>
<th>Upper-extremity fracture group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Active intervention</td>
<td>Placebo</td>
</tr>
<tr>
<td></td>
<td>(n = 11)</td>
<td>(n = 18)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>77 ± 6</td>
<td>72 ± 10</td>
</tr>
<tr>
<td>Age at menopause (y)</td>
<td>50 ± 4</td>
<td>48 ± 5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.6 ± 4.0</td>
<td>23.3 ± 4.4</td>
</tr>
<tr>
<td>Daily calcium intake (mg)</td>
<td>815 ± 301</td>
<td>645 ± 232</td>
</tr>
<tr>
<td>Smoking (pack-years)²</td>
<td>19.2 ± 23.9</td>
<td>18.8 ± 27.1</td>
</tr>
<tr>
<td>Alcohol intake (units)²</td>
<td>3.5 ± 5.2</td>
<td>2.2 ± 3.6</td>
</tr>
<tr>
<td>PINP (µg/L)</td>
<td>74.6 ± 32.3</td>
<td>94.6 ± 44.6</td>
</tr>
<tr>
<td>OC (ng/mL)</td>
<td>16.9 ± 8.2</td>
<td>21.1 ± 12.9</td>
</tr>
<tr>
<td>ICTP (µg/L)</td>
<td>11.7 ± 4.3</td>
<td>13.1 ± 6.3</td>
</tr>
<tr>
<td>25(OH)D (nmol/L)</td>
<td>33.1 ± 16.7</td>
<td>39.9 ± 16.8</td>
</tr>
<tr>
<td>PTH (pmol/L)³</td>
<td>3.4 ± 1.0</td>
<td>4.1 ± 1.6</td>
</tr>
<tr>
<td>BMD L2-L4 (g/cm²)</td>
<td>0.899 ± 0.239</td>
<td>0.809 ± 0.186</td>
</tr>
<tr>
<td>BMD hip, total (g/cm²)</td>
<td>0.573 ± 0.080</td>
<td>0.618 ± 0.130</td>
</tr>
<tr>
<td>Timed Up &amp; Go test (s)</td>
<td>33.9 ± 18.3</td>
<td>34.4 ± 14.6</td>
</tr>
</tbody>
</table>

*All values are ± SD. PINP, N-terminal propeptide of type I collagen; OC, osteocalcin; ICTP, C-terminal telopeptide of type I collagen; 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone; BMD, bone mineral density. For data with a skewed distribution, a normal distribution was obtained by log transformation.

² P for the difference between the hip and upper-extremity fracture groups by independent-sample t test, P < 0.05.

³ Significantly different from the active group, P < 0.05.

⁴ Skewed distribution.
No significant difference was found between the intervention groups after at any visit (Figure 1E).

Upper-extremity fracture group. To account for differences present already at baseline, we included baseline as a covariate in a repeated-measures analysis of variance (mixed model) and found the difference between treatments to remain significant (P < 0.05). Serum PINP was significantly lower in the active intervention group than in the placebo group after 1, 3, and 12 mo (P < 0.01; Figure 2C).

Serum osteocalcin was significantly lower at 1, 3, and 12 mo in the intervention group than in the placebo group (P < 0.01), and the change compared with baseline was significant for in active intervention group (P < 0.001) but not in the placebo group (Figure 2D).

ICTP declined in both intervention groups and was significantly different from baseline after 12 mo in both groups (P < 0.05). No significant difference between intervention groups was found (Figure 2E). For all patients, bone resorption evaluated by ICTP correlated with the level of physical mobility evaluated by the timed Up & Go test. Patients who took longer to complete the test had a higher level of resorption (R² = 0.423, P < 0.01, r = 0.651). Serum concentrations of 25(OH)D and PTH and age were not significantly correlated with the level of bone resorption.

Bone mineral density

BMD for the lumbar spine scanning procedures with and without the legs elevated showed that the BMD values with the legs elevated were 1 ± 1.78% lower than the values without the legs elevated. Both in a per-protocol analysis and an intention-to-treat
analysis, intervention with calcium and vitamin D resulted in a significant increase in BMD of the lumbar spine after 12 mo compared with baseline values for the intervention group, whereas BMD of the lumbar spine after 12 mo had decreased compared with baseline in the placebo group. No significant changes were shown for BMD of the hip. The results are shown in Table 2.

In a regression analysis, age had an effect on change in BMD for both scanning regions (age × hip BMD, \( P < 0.01 \), and age × lumbar spine BMD, \( P < 0.05 \)). Stratification according to age showed a more pronounced effect of treatment (lumbar spine BMD) in the patients aged ≤70 y than in those aged >70 y (0.993 ± 0.131 compared with 0.868 ± 0.216; \( P < 0.05 \)). Lumbar spine BMD increased in the group aged ≤70 y and decreased in the age group aged >70 y. BMD values stratified by age and intervention group are shown in Figure 3. No significant differences were found between subgroups for the hip region.

**DISCUSSION**

In old age, the amount of bone resorption exceeds the amount of bone formation, which results in a decline in bone quality and bone strength with a subsequently increased risk of fracture (15, 16). Deficiency in vitamin D worsens the condition by reducing active intestinal calcium absorption and increasing bone resorption to mobilize calcium to the circulation (17).

In the present study, patients with a fracture of the hip had higher bone turnover, lower BMD, and lower vitamin D concentrations and demonstrated poorer physical performance than did patients with upper-extremity fractures. The dropout rate was...
also significantly higher for the hip fracture patients, which indicates that these patients are generally frailer and have poorer bone status than do patients with fracture of the upper extremity (18).

Intervention with both 1400 IU cholecalciferol and 200 IU cholecalciferol resulted in a significant increase in serum 25(OH)D; the larger dose resulted in the greatest increase in 25(OH)D. Patients with a hip fracture showed the greatest benefit: 25(OH)D increased from an insufficient concentration of 33 nmol/L to 85 nmol/L. Intervention with calcium and vitamin D also increased the daily intake of elementary calcium to 1550 mg, which may be responsible for the concomitant decline in PTH. Baseline concentrations of PTH were lower than expected in a vitamin D–deficient population. Physical activity has been shown to correlate with bone resorption and level of PTH. The sudden immobilization of these patients may release calcium from bone and be the cause of the relative low PTH concentration at baseline (19, 20).

In animal studies, a resistance to IGF-I at the cellular level has been proposed as a possible mechanism causing bone loss during immobilization (21). Other theories consider an altered communication between bone cells or altered gene expression (22, 23). The strong effect of immobility on bone remodeling has also been described in clinical studies. Calcium is released from bone when patients are immobilized and prevents secondary hyperparathyroidism, even though the patients are vitamin D deficient (24, 25). The osteonecrosis in relation to the fracture episode and the subsequent immobilization causes an initial increase in bone resorption. This is succeeded by an increase in bone formation as the result of both fracture healing and incipient mobilization (11). The higher level of bone resorption found in the hip fracture patients correlated with a lower physical mobility status independently of serum concentrations of 25(OH)D and PTH. Intervention with calcium and vitamin D for 12 mo significantly reduced the level of bone formation markers in both fracture groups, whereas concentrations of bone resorption markers were reduced significantly only in the upper-extremity fracture group.

PINP and osteocalcin are both markers of bone formation, but whereas PINP decreased after the fracture episode, osteocalcin increased. An increase in osteocalcin after a fracture episode was reported in previous studies (26). Osteocalcin is cleaved from the procollagen molecule during formation and is incorporated into bone during mineralization. Osteocalcin is also released from bone during resorption. It has been argued that the molecule is partly metabolized during this process and that, therefore, osteocalcin cannot be considered a bone turnover marker, because these metabolized products are expected to be cleared by the kidneys and not evaluated in the assays (27). Differences in osteoid volume could result in corresponding differences in

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**TABLE 2**

Changes (Δ) in bone mineral density (BMD) after 12 mo of intervention in both hip and upper-extremity fracture patients

<table>
<thead>
<tr>
<th></th>
<th>Intervention group</th>
<th>Placebo group</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD (g/cm²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per-protocol analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔLBMD (g/cm²)</td>
<td>0.006</td>
<td>–0.012²</td>
</tr>
<tr>
<td>ΔHBMD (g/cm²)</td>
<td>0.006</td>
<td>0.002</td>
</tr>
<tr>
<td>Intention-to-treat analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔLBMD (g/cm²)</td>
<td>0.004</td>
<td>–0.008²</td>
</tr>
<tr>
<td>ΔHBMD (g/cm²)</td>
<td>–0.001</td>
<td>–0.001</td>
</tr>
</tbody>
</table>

1 Absolute changes in BMD are displayed for the 2 treatment groups. Statistical comparison of the treatment effect between intervention groups was analyzed by using an independent-sample t test in both a per-protocol and an intention-to-treat analysis. ΔBMD, change in BMD at the lumbar region from 12 mo to baseline; ΔHBMD, change in BMD at the hip region from 12 mo to baseline.

2 Significantly different from the intervention group, P < 0.05.

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**FIGURE 3.** Changes in bone mineral density (BMD) of the total hip region and lumbar spine after 1 y stratified by age group: ≤70 y or >70 y old. A significant age-by-treatment interaction was found for both variables. The results of the statistical comparison between the intervention group and the placebo group within age subgroups are denoted by an asterisk. *P < 0.05.
serum concentrations of osteocalcin. Biopsy studies have shown a correlation between osteoid volume and serum osteocalcin concentration (28). A more optimal supply of calcium and vitamin D and a consequently better environment for mineralization of bone in the intervention group may reduce osteoid volume and explain the lower osteocalcin concentration in the intervention group.

In the present study, lumbar spine BMD was evaluated without leg elevation, and this method resulted in a 1% overestimation of BMD, which may result in an underestimation of the effect of treatment. Lumbar BMD increased significantly for patients in the active treatment group aged 70 y. The changes in BMD correlated with the level of physical activity, ICTP, and age. Because frail patients older than 70 y of age are immobile, they are not able to decrease bone resorption and increase bone mass when treated with calcium and vitamin D. The importance of sufficient mechanical loading has been shown in clinical trials; the effect of intervention with calcium and vitamin D is reduced if patients are immobile (29, 30).

Because of higher precision and higher metabolic activity, a treatment effect is detected earlier in the lumbar spine region than in the hip region. In our study, however, no significant effect of treatment was detected in the hip region (31). This could be explained by a lack of power. Recent, large randomized clinical trials failed to show an effect of calcium and vitamin D supplementation on BMD and fracture rates (32, 33). The studies were large and well designed with an intention-to-treat approach in their analysis. The lack of a significant effect of intervention may have been due to poor compliance in the study populations and participant dropout. The use of an intention-to-treat approach may under these circumstances mask the effect of intervention, but gives a more correct picture of what we can expect when a population is treated.

If lack of compliance and drop out is a great problem in these elderly populations, a different strategy may be necessary in which more attention is given to obtaining better compliance. We showed that in a population of women and men with a recent low-energy fracture, it is possible to obtain an increase in serum 25(OH)D of 200% to a concentration of 85 nmol/L when a great deal of attention is paid to compliance.

In conclusion, the hip fracture patients had poorer bone status, vitamin D deficiency, and lower physical performance than did the upper-extremity fracture patients. Both in a per-protocol analysis and in an intention-to-treat analysis, calcium and vitamin D supplementation for 1 y increased vitamin D status and BMD in the lumbar spine region, especially for those younger than 70 y of age. The biochemical markers of bone remodeling increased around 1 mo after fracture and declined thereafter. Supplementation with calcium and vitamin D is important for patients with low-energy fractures because it decreases bone loss. The effect of intervention was positively related to physical performance, which emphasizes the importance of mobilization.

Blinding, coding, and donation of tablets by Pharma Vinci A/S, Frederiks-vaerk, Denmark is acknowledged with appreciation. We are grateful to Lise Martinussen’s excellent work in the unit with the care of the patients and with sample analysis. Special acknowledgment also goes to associate professor Lene T Skovgaard for her statistical assistance.

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Safe gluten threshold for patients with celiac disease: some patients are more tolerant than others

Dear Sir:

The long-expected first randomized controlled study of a micro-gluten challenge in patients with celiac disease has been published by Catassi et al (1). The authors stated that 50 mg gluten/d is harmful in celiac disease patients, whereas 10 mg/d is not. The results are based on a small but significant change in the small-intestinal mucosal villous height crypt depth ratio, even though the biopsy specimens did not show mucosal cell infiltrativity or accumulation of inflammatory cells—the known first effects of the disease on gluten ingestion. The question of what happens at doses between 10 and 50 mg/d (eg, a dose of 25 mg/d) remains.

A diet entirely free of gluten contamination would of course be ideal, but today it seems to be unrealistic; even naturally gluten-free products may contain significant amounts of gluten (2). Furthermore, too strict limits might lead to the poor availability of gluten-free products, which again would hamper overall dietary compliance. The study by Catassi et al also implies that minor gluten contamination was not harmful to most of the patients.

In Finland, the current Codex standard of 200 ppm (mg/kg) seems to not be too high; at this dose, mucosal recovery is complete and the quality of life of celiac disease patients is not different from that of the general population (3). In fact, the mean ratio of villous height to crypt depth was higher than in the study by Catassi et al, =3.2 and 2.5, respectively (4). Because most of our gluten-free products contained <200 ppm, we concluded that 100 ppm would be a safe limit (2). The daily ingestion of gluten might also then remain well below 50 mg, at least in Finland.

Clearly, more studies are needed to settle on a safe limit of gluten contamination in gluten-free products. In the meantime, what are the clinical implications of the current studies? It appears that even occasional dietary lapses may slow down or prevent mucosal recovery (3, 4). Whether the safe limit of gluten contamination should be 0, 20, 50, or 100 ppm remains to be seen. As the study by Catassi et al (1) showed, celiac disease patients respond individually to small amounts of gluten. The individual variability denotes that the treatment should be individual too. Here, we emphasize the role of a control biopsy sample taken from adult celiac disease patients consuming a gluten-free diet. Although a biopsy is not usually required to establish a diagnosis, it is important to confirm mucosal integrity. A clear improvement in mucosal integrity after 1 y of a diet indicates that the diet is gluten-free enough to render mucosal recovery. In conclusion, the results of this interesting pilot study need confirmation before firm conclusions can be drawn about the safe limit of gluten ingestion.

The authors had no conflicts of interest to declare.

Pekka Collin

REFERENCES

Reply to P Collin et al

Dear Sir:

We sincerely appreciate the comments from Collin et al and agree with most of the issues raised. Indeed, ours was the first randomized controlled double-blind study that was aimed at settling a long discussion among experts in the field on a safe threshold of traces of gluten allowable for celiac disease patients (1). Given the complexity of the study design, we had to limit our microchallenge doses to 10 and 50 mg gluten/d. Therefore, we surely agree that information regarding intermediate amounts of gluten are lacking. Nevertheless, the fact that 10 mg gluten/d appeared safe for most of the subjects studied, whereas 50 mg/d induced histologic changes after 3 mo of exposure, allowed us to conclude that 20 ppm would be the safest and most conservative threshold to recommend for gluten-free products. Given the regional differences in daily wheat substitute intakes, this threshold would be applicable worldwide rather than being customized to local lifestyle realities. The fact that the circulation of gluten-free products is now beyond national boundaries requires guidelines as widely applicable as possible. We agree that too strict limits, as advocated by some celiac support leaders (2), may lead to the limited availability of gluten-free products and an increase in production...
Evidence-based medicine and vitamin E supplementation

Dear Sir:

In a recent editorial in the Journal, Traber (1) recommended vitamin E supplementation for most adults in the United States. The logic behind her recommendation was as follows. First, Wright et al (2) reported in the same issue of the Journal that the lowest overall risk for mortality in the 19-y follow-up of the Alpha-Tocopherol Beta-Carotene (ATBC) Study occurred at serum vitamin E concentrations of 13–14 mg/L, and Traber labels that as an optimal concentration for reducing the risk of chronic disease. Second, 75% of men in the United States have serum vitamin E concentrations of <14.6 mg/L, which suggests widespread vitamin E deficiency in her opinion. Third, “given the dietary habits of most Americans,” “optimal” concentrations of serum vitamin E are achievable only with vitamin E supplements (1).

We believe that Traber’s recommendation for vitamin E supplementation in the general population is unjustified. Inferring cause and effect and making such broad public health recommendations for supplements on the basis of observational data violate the established principles of evidence-based medicine. In fact, her recommendations are not aligned with those based on systematic reviews of large clinical trials of vitamin E supplementation, which do not recommend vitamin E supplement use (3) and discourage the use of high-dose vitamin E supplements (4).

Other clinical outcomes reported from the ATBC Study show that supplementation with 50 mg vitamin E/d has divergent relations with the incidence of pneumonia and the common cold. Although vitamin E showed no overall benefit against pneumonia, the age at smoking initiation significantly modified the effect of vitamin E, so that it was harmful or beneficial, depending on this characteristic in each participant (6). The effect of vitamin E on common cold incidence was significantly modified by smoking level at baseline, age, and residential neighborhood (7). It is worth noting that, in both of these cases, smoking-related variables modified the effect of vitamin E. Although it is not reasonable to assume that the factors that modify the effect of vitamin E on respiratory infections identically modify the effect of vitamin E on cancer, coronary heart disease, or total mortality, the possibility that the effect on these latter outcomes is also modified by various factors should not be ignored. Because of this heterogeneity in the effects of vitamin E, it is possible that supplementation of a wide population may cause harm to some restricted population groups, as indicated by a recent meta-analysis (4).

These results highlight the misconception that supplementing to correct “deficiencies” of a single micronutrient is an inaccurate interpretation of the relation between nutritional markers and the risk of chronic disease in epidemiologic studies. Most blood concentrations of micronutrients, including antioxidants, are collinear. High concentrations of antioxidants reflect an antiatherogenic diet (lower in fat and saturated fat and higher in fruit, vegetables, nuts, whole grains, and low-fat dairy), which also has beneficial effects on traditional cardiovascular disease risk factors, including blood pressure, lipid concentrations, and glucose metabolism. Supplementation with vitamin E has no effect on traditional cardiovascular disease risk factors and does not lower the risk of chronic disease by other proposed mechanisms, such as by reducing oxidative stress.
Traber (1) argued that 93% of men and 96% of women in the United States do not consume the recommended amount of vitamin E. However, the current US recommendation for vitamin E is based on peroxide-dependent erythrocyte hemolysis, a surrogate endpoint that has not been validated against any clinically relevant outcome (8, 9). Furthermore, according to the current nutritional recommendations, there is no evidence that, among free-living persons, dietary vitamin E intake may meaningfully correlate with plasma α-tocopherol concentrations (8). We are not aware of any reasonable evidence indicating that 93% of men and 96% of women in the United States may suffer any harmful effect on health because of their “low” vitamin E intake.

In our opinion, the attitude toward vitamin E supplementation should be based on randomized controlled trials, which have not shown a benefit in preventing or treating chronic diseases, and not on observational studies, which are highly susceptible to biases that may remain even after statistical adjustment for confounders (5, 10). Although it is possible that some population groups may benefit from vitamin E supplementation, the evidence is so equivocal that it is inappropriate to make the sweeping recommendation for vitamin E supplementation in the United States that Traber makes. Implying health benefits of supplementation in the general population is contrary to the evidence; moreover, it puts people at risk if excess use occurs and will benefit only the industry that produces, promotes, and protects the continued sale of supplement products.

Neither author had a personal or financial conflict of interest with respect to the study by Wright et al or the editorial by Traber.

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Reply to H Hemilä and ER Miller III

Dear Sir:

We appreciate the earlier editorial by Traber (1) and the current comments from Hemilä and Miller. In our study, we found that higher prerandomization serum concentrations of α-tocopherol were associated with significantly lower total and cause-specific mortality in men participating in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study (2). Only 10% of participants reported vitamin E supplement use before randomization, and the exclusion of these men from our analyses did not alter the observed relations. This indicates that prettrial serum vitamin E concentrations in the ATBC Study population were achieved primarily through dietary intakes and other host factors known to affect circulating vitamin E concentrations (eg, age, body mass index, and serum cholesterol) and not through vitamin E supplement use. It is important to note that neither the use of supplemental vitamin E before the trial nor the trial intervention itself (50 mg all-rac-α-tocopheryl acetate) was the focus of our report.

As Traber (1) pointed out in her editorial, we observed the lowest overall mortality at serum α-tocopherol concentrations of ≈13 mg/L (14 mg/L for cardiovascular disease mortality; see Figure 2 in reference 2). It should be emphasized that mortality did not diminish further at higher concentrations: relative mortality estimates drifted back toward unity (relative risk = 1) as blood concentrations rose beyond 13–14 mg/L. The precise vitamin E intake required to achieve this “optimum” serum concentration cannot be inferred from our study, however. Even though men in the fourth quintile of serum vitamin E intake were associated with significantly lower total and cause-specific mortality, we did not find a clear concentration–response relationship for lower serum vitamin E concentrations. A range of serum concentrations can result from any single daily dietary intake and, conversely, that a range of intakes can lead to a single target blood or tissue concentration. Carefully controlled feeding studies can help shed light on the amounts of vitamin E that need to be ingested to achieve particular blood concentrations. In this regard, however, studies have made clear that a range of serum concentrations can result from any single daily dietary intake and, conversely, that a range of intakes can lead to a single target blood or tissue concentration. Finally, it should be reemphasized that any “optimal” serum α-tocopherol value that we observed with respect to overall mortality among Finnish male smokers may not be applicable in other groups, including nonsmokers, women, and ethnically diverse populations. This question should be addressed in other studies.

Traber correctly highlights the possibility that dietary recommendations based on preventing overt deficiency symptoms—peroxide-dependent erythrocyte hemolysis, in the case of vitamin E—may
differ from recommendations based on the prevention of chronic disease or death. As she notes, the Recommended Dietary Allowance (RDA) for vitamin E is 15 mg α-tocopherol/d for men and women >18 y old, and this amount is based on experiments conducted almost a half-century ago in men who were experimentally vitamin E depleted (3). Overt vitamin E deficiency is extremely rare in the United States, despite the fact that most US men and women are not meeting the dietary recommendation for vitamin E. Again, additional research aimed at clarifying the optimal serum concentrations of vitamin E for chronic disease prevention in multiple populations, as well as the amount of dietary vitamin E required to achieve those concentrations, will be informative. As more data accumulate, the RDAs for vitamin E may need to be reevaluated with respect to important public health endpoints such as chronic disease risk and mortality and not only in relation to the avoidance of deficiency states.

We agree with Hemilä and Miller that populationwide vitamin E supplementation is not warranted at this time, according to the available research. This body of evidence includes both a demonstrated lack of efficacy for overall mortality in several supplementation trials and the elevated mortality suggested—but not universally accepted (4)—by a recent meta-analysis for high-dose vitamin E supplementation (5). We explicitly state in our report, “Because supplemental vitamin E has not been shown to reduce mortality in randomized trials, efforts to improve vitamin E status through dietary means (eg, through increasing consumption of foods rich in vitamin E, including nuts, seeds, whole grains, and dark-green leafy vegetables) may be warranted, particularly if future prospective studies show similar serum mortality associations in diverse populations, including nonsmokers” (2). Although, as Traber suggests, vitamin E–rich food sources have traditionally been of limited popularity in the American diet, we support dietary modification rather than supplementation at this time.

Results from well-designed prospective cohort studies have made, and will continue to make, substantial contributions to our knowledge regarding micronutrient-disease relationships, even when the research findings appear to contradict those from controlled trials. A case in point is the diametric opposition of the conclusions of the original ATBC Study findings for β-carotene and lung cancer (6) to the findings from most case-control and cohort studies available at the time (7). We must remain cognizant of the fact that observational studies and clinical trials often address different questions. For example, trials typically test the efficacy of single-nutrient supplements, at various dosages and administered over several years, whereas observational studies examine the associations between habitual dietary intake (or serum concentrations) of nutrients that are derived primarily from foods, which contain many other, potentially anticarcinogenic substances. Whereas it is true that observational studies are susceptible to confounding and measurement error and that trials are typically free from such biases, we believe that recommendations regarding supplement use should be based on the totality of evidence provided by basic experimental and epidemiologic studies, as well as by randomized controlled trials.

None of the authors had any personal or financial conflict of interest.

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LETTERS TO THE EDITOR

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REFERENCES

Reply to H Hemilä and ER Miller III

Dear Sir:

My recent editorial in the Journal (1) emphasized the difficulty in setting the daily α-tocopherol requirement and was not intended as advocacy for high-dose vitamin E supplementation. Evidence-based medicine is not based only on randomized clinical trials (RCTs) and meta-analyses but also takes into account all relevant evidence. The scientific evidence that vitamin E is essential for human health is overwhelming. α-Tocopherol insufficiency results in a sensory neuropathy, which has been documented in patients with ataxia and vitamin E deficiency (AVED). Symptoms are secondary to a genetic defect in the hepatic α-tocopherol transfer protein (α-TPP) (2). The plasma α-tocopherol concentrations of persons with AVED are one-tenth of normal, and their nerves become α-tocopherol–depleted before symptom onset (3); α-tocopherol supplements reverse or halt symptom progression (2). Thus, the nervous system is vulnerable to inadequate α-tocopherol status.

Hemilä and Miller refer to peroxide-dependent erythrocyte hemolysis as “a surrogate endpoint that has not been validated against any clinically relevant outcome.” However, more than 30 y ago, this test was used clinically to show that children with cystic fibrosis were vitamin E deficient (4). These children absorbed vitamin E poorly and thus had low plasma α-tocopherol concentrations, anemia, and increased erythrocyte turnover—symptoms that were reversed by α-tocopherol supplements (4). The Food and Nutrition Board (FNB) used peroxide-dependent erythrocyte hemolysis data to set the current recommended dietary allowance (RDA)—15 mg

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alpha-tocopherol (5)—which is lower than the “current US recommendation for vitamin E” cited by Hemilä and Miller. The US RDA uses the daily value (DV), which is defined from the 1968 FNB recommendation (also based on erythrocyte hemolysis) of 30 IU (30 mg dl-alpha-tocopheryl acid); %DV is used on food labels.

Hemilä and Miller stated, “Most blood concentrations of micronutrients, including antioxidants, are collinear.” This statement is incorrect with respect to vitamin E. An appreciation of the complex pharmacokinetics of alpha-tocopherol is essential to understanding its disposition and human vitamin E status. High plasma alpha-tocopherol concentrations may reflect high alpha-tocopherol intakes. However, hyperlipidemia also elevates plasma alpha-tocopherol, because alpha-tocopherol concentrations are collinear with circulating lipids. In normolipidemic subjects, low plasma alpha-tocopherol concentrations reflect inadequate vitamin E intakes. When inadequate amounts of alpha-tocopherol are consumed, plasma concentrations are maintained by alpha-TTP, whereas peripheral tissue alpha-tocopherol depletion occurs (3). To assess vitamin E status, one should measure plasma alpha-tocopherol and lipid concentrations and, ideally, tissue alpha-tocopherol concentrations. Alpha-Tocopherol is not found in most high-antioxidant foods, such as fruit and vegetables. Low-fat diets decrease alpha-tocopherol intakes because the fat-soluble vitamin is largely present in high-fat foods. Therefore, substantial changes in the kinds of foods Americans eat are needed for them to obtain 15 mg alpha-tocopherol/d from dietary sources, such as seeds, nuts, spinach, and safflower oil.

What is the downside to consuming a less-than-optimal alpha-tocopherol intake? It is difficult to determine, because it takes decades for symptoms of suboptimal vitamin E status to become readily apparent. It took ≈40 y for symptoms to be detectable in a patient with chronic fat malabsorption and alpha-tocopherol deficiency (7). Such a delay in the first appearance of symptoms shows the fallacy of concluding, after an observation of only a relatively short time (eg, 5 y), that there is no harm to inadequate vitamin E intakes. The Alpha-Tocopherol Beta-Carotene Cancer Prevention (ATBC) Study provides an interesting contrast. Analysis of baseline serum alpha-tocopherol concentrations in ≈29 000 men, nearly one-half of whom are now dead, showed a significant correlation between high serum alpha-tocopherol status and lower chronic disease mortality (8), which suggested that long-term dietary habits that provide higher alpha-tocopherol intakes are beneficial. In contrast, supplementation for only 5–8 y with 50 mg dl-rac-alpha-tocopheryl acetate (22 mg 2R-R-alpha-tocopherol or ≈1.5 times the RDA) showed no such relation (8). Given that clinical symptoms take decades to appear in humans with various chronic diseases, the effects of correcting suboptimal vitamin E intakes cannot be assessed by using RCTs that last only years, rather than decades. Therefore, the suggestion by Hemilä and Miller to carry out RCTs seems impractical, if not unethical, given the potential for inadequate alpha-tocopherol intakes in the “placebo” group to deplete tissue, especially nervous system tissue, of alpha-tocopherol.

Hemilä and Miller contend that high-dose alpha-tocopherol is dangerous, but they specify no mechanism for any adverse effect. Miller et al (9), in a meta-analysis analyzing the relation between dose and mortality, found a benefit of ≈4% when vitamin E supplements were provided in the range of dietary requirements. This outcome contradicts their widely publicized claim of vitamin E supplement harm, a claim that was criticized in many letters to the editor in the journal that published the report of Miller et al (see the July 2005 issue of Annals of Internal Medicine). A systematic review sponsored by the National Institutes of Health concluded that the evidence was insufficient to prove the “presence or absence of benefits” for vitamin E supplements (usually ≥400 IU) for the prevention of cancer or chronic disease (10). The Cache County Study found that vitamin E supplements had “no effect” on mortality, but their conclusion was based on a combination of outcomes “[in which] increased mortality was observed in subjects with severe cardiovascular disease and a possible protective effect in those without” (11). This latter finding is of interest because the Women’s Health Study, a primary prevention trial with vitamin E supplements (600 IU every other day for 10 y) in ≈40 000 healthy women, concluded that vitamin E had no effect on the occurrence of heart disease or cancer (12). However, in subgroup analysis, vitamin E supplements decreased cardiac mortality by 49% in women ≥65 y old—ie, those who are at greater risk of heart disease than are younger women (12). Taken together, these studies suggest that, in healthy persons, a generous alpha-tocopherol intake for a prolonged period is beneficial, not harmful. Thus, intakes in the range of the RDA—15 mg alpha-tocopherol/d—obtained from a healthy diet, from a multivitamin, or as an alpha-tocopherol supplement, appear to me to be a prudent public health recommendation.

The author had no personal or financial conflict of interest.

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REFERENCES
Cognitive impairment in older Americans in the age of folic acid fortification

Dear Sir:

The recent article by Morris et al (1) reports perplexing findings about the association of folic acid, vitamin B-12, and cognitive functioning among healthy participants aged ≥60 y in the United States from the National Health and Nutrition Examination Survey (NHANES) 1999–2002. They found that participants with a combination of high serum folate concentrations (>59 nmol/L) and normal vitamin B-12 status were less likely to have cognitive impairment, whereas those with a combination of high serum folate and low vitamin B-12 status were more likely to have cognitive impairment. The article by Morris et al and the accompanying editorial by Smith (2) suggest that these findings have important implications for current folic acid fortification practices in the United States and for other countries considering folic acid fortification. Although the analysis is well done, the lack of sufficient information about the small subgroup of interest (n = 42) with high serum folate and low vitamin B-12 status, the cross-sectional nature of the data, and the lack of consideration of the relative contribution of different sources of folic acid, which include supplements and all fortified food products, including ready-to-eat (RTE) breakfast cereals, raise important questions that should be considered when drawing inferences from this study. In countries in both the developed and developing world, it is likely that the only significant source of folic acid is fortified flour. The findings of Morris et al might only be applicable to seniors in the United States whose use of supplements and consumption of folic acid–fortified RTE breakfast cereals is high.

It would have been useful to see a comparison between participants with high serum folate and normal serum folate. It would have been more useful to see a comparison between participants in the highest folate quintile with low and normal vitamin B-12 status. The subgroup with high serum folate and low vitamin B-12 status was a highly restricted population: 1.1% (42/3706) of the serum survey participants, 2.5% (42/1684) of eligible seniors, and only 3.2% (42/1302) of those who could have been evaluated for cognitive function. The analytic and reporting guidelines of NHANES state that, “The more one deviates from the original analytic categories . . . the more important it is to evaluate the results carefully and to interpret the findings cautiously” (3). Therefore, drawing appropriate inferences from this small subgroup might be problematic.

Another potential limitation of the study by Morris et al is inherent in any study in which the use of folic acid is not randomized. When blood folate concentrations and/or intakes of folic acid–fortified food products are used to create folate and folic acid exposure strata, most subjects in the highest stratum will be supplement users and most subjects in the lowest stratum will not be supplement users. To illustrate this stratification among seniors in the United States, we divided non-Hispanic white participants aged ≥60 y from NHANES (2001–2002) into serum folate quintiles and analyzed their use of supplements and their dietary intake data (4). We used SUDAAN (version 8.1; Research Triangle Institute, Cary, NC) to take into account the complex survey design and calculated for each quintile of serum folate the proportion of non-Hispanic white seniors who used a supplement containing folic acid, the geometric mean of serum folate, and the total daily intake of folic acid from supplements and food. We also calculated the total daily intake of synthetic folic acid from all fortified foods and estimated the total daily intake of enriched cereal-grain products by removing folic acid found in RTE breakfast cereals from all fortified foods. Finally, we calculated the proportion of supplements containing folic acid that also contained vitamin B-12 and the median content of vitamin B-12 in those supplements.

It is shown in Table 1 that 92% and 12% of non-Hispanic white seniors in the highest and lowest folate quintiles, respectively, used supplements containing folic acid. As documented in the recent National Institutes of Health State-of-the-Science conference on multivitamin-mineral supplement use and chronic disease prevention, there appear to be important behavioral and health-related differences between individuals who report using a supplement (eg, elderly persons, those with higher education, those with higher income, those with healthier lifestyles and diets, and those who are seeking to prevent a serious disease) and those who do not report using a supplement (5). Such differences increase the possibility that unmeasured confounding related to unique differences between subjects who take supplements and those who do not could account, in part, for the findings reported by Morris et al.

Of all the non-Hispanic white seniors, the 80th percentile of serum folate, which defined our highest quintile, was 58.2 nmol/L, which was similar to the value (59 nmol/L) Morris et al used to define their highest quintile. It is also shown in Table 1 that, for non-Hispanic white seniors in the highest quintile, the mean serum folate concentration was 77.9 nmol/L, and the geometric mean daily total intake of folic acid from fortified food products plus supplements was 555 μg. Mean daily intakes of folic acid from all fortified food products and enriched cereal-grain products were 144 and 86 μg, respectively. In the highest folate quintile, daily intakes of folic acid from all fortified food products constituted 26% (144/555), and enriched cereal-grain products constituted only 15% (86/555) of the total daily intake of folic acid. It is unclear why both the article by Morris et al and the accompanying editorial focus most of their discussion on folic acid fortification. Only folic acid from enriched cereal-grain products should be considered when making inferences that affect other countries where folic acid fortification of flour is likely the only source of synthetic folic acid in the diet.

In addition, we used SAS (version 9.1; SAS Institute Inc, Cary, NC) to evaluate correlations with serum folate concentrations in our table. Across the entire serum folate distribution, we found a strong correlation between the serum folate concentration and both the total daily intake of folic acid (r = 0.293, P < 0.0001) and the daily use of supplements containing folic acid (r = 0.289, P < 0.0001). We found a very weak, but statistically significant, correlation between the serum folate concentration and the total daily intake of folic acid from all fortified food products (r = 0.080, P = 0.016), but found no correlation with enriched cereal-grain products (r = −0.026, P = 0.444). These data clearly show that high concentrations of serum folate in older adults are not due to the consumption of enriched cereal-grain products and suggest that the findings of Morris et al should not be considered as relevant outside of the United States.

Another intriguing question about the Morris et al study concerns the subgroup of seniors with high serum folate and low vitamin B-12 status. In NHANES (2001–2002), we found that 92% of non-Hispanic white seniors in the highest serum folate quintile took supplements containing folic acid and that 97% (95% CI: 90%, 99%) of these supplements also contained vitamin B-12; the median daily vitamin B-12 content was 16.6 μg (95% CI: 6.8, 23.1 μg). Therefore, it is unclear why these 42 participants continued to have low vitamin B-12 status when almost all of them would have been consuming oral vitamin B-12. Could they have been unable to absorb vitamin B-12? Could they have been developing pernicious anemia? Could their vitamin B-12 status when almost all of them would have been consuming oral vitamin B-12? These are some of the questions that need to be addressed before the finding of an association between high serum folate and cognitive impairment can be generalized to the entire population of US
TABLE 1
Proportion of participants who used supplements containing folic acid, serum folate concentrations, total daily intake of folic acid, and total daily intake of folic acid from all folic acid–fortified food products and enriched cereal-grain products by serum folate quintiles in non-Hispanic white seniors aged ≥60 y from the National Health and Nutrition Examination Survey (NHANES), 2001–2002.

<table>
<thead>
<tr>
<th>Serum folate quintiles</th>
<th>Percentage of participants who used supplements containing folic acid</th>
<th>Serum folate (^2)</th>
<th>Daily intake of folic acid from all fortified food products plus supplements (^2)</th>
<th>Daily intake of folic acid from all fortified food products (^2)</th>
<th>Daily intake of enriched cereal-grain products (^2,3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>(\text{nmol/L})</td>
<td>(\mu g/d)</td>
<td>(\mu g/d)</td>
<td>(\mu g/d)</td>
</tr>
<tr>
<td>Highest ((n = 179))</td>
<td>92 (87, 95)</td>
<td>77.9 (75.4, 80.4)</td>
<td>555 (484, 627)</td>
<td>144 (135, 154)</td>
<td>86 (78, 94)</td>
</tr>
<tr>
<td>High ((n = 190))</td>
<td>74 (64, 83)</td>
<td>48.9 (48.3, 49.5)</td>
<td>409 (358, 460)</td>
<td>130 (111, 149)</td>
<td>81 (71, 92)</td>
</tr>
<tr>
<td>Middle ((n = 174))</td>
<td>46 (38, 54)</td>
<td>38.4 (37.9, 39.0)</td>
<td>274 (221, 326)</td>
<td>142 (120, 164)</td>
<td>91 (77, 105)</td>
</tr>
<tr>
<td>Low ((n = 182))</td>
<td>22 (16, 29)</td>
<td>28.7 (28.4, 29.1)</td>
<td>166 (138, 194)</td>
<td>116 (99, 134)</td>
<td>92 (81, 104)</td>
</tr>
<tr>
<td>Lowest ((n = 178))</td>
<td>12 (7, 20)</td>
<td>17.9 (16.7, 19.0)</td>
<td>125 (102, 148)</td>
<td>98 (80, 117)</td>
<td>82 (68, 97)</td>
</tr>
<tr>
<td>Total ((n = 903))</td>
<td>49 (45, 54)</td>
<td>37.9 (35.9, 39.6)</td>
<td>266 (241, 292)</td>
<td>125 (113, 137)</td>
<td>87 (79, 94)</td>
</tr>
</tbody>
</table>

\(^1\) 95% CIs in parentheses.

\(^2\) All values are geometric means.

\(^3\) Folic acid from all fortified food after the removal of folic acid found in ready-to-eat breakfast cereals.

seniors with low vitamin B-12 status. We agree with Morris et al that further study is necessary to clarify their findings.

Morris et al also attribute the presence today of unmetabolized (free) folic acid in blood to folic acid fortification. Kelly et al (6) showed that consumption of a supplement containing 200 \(\mu g\) folic acid would produce free folic acid. If free folic acid in the blood were responsible for any adverse effects, such effects, if detectable, would have been present before folic acid fortification began in the United States. Any person using a supplement containing folic acid should have measurable blood concentrations of free folic acid. The overall prevalence of dietary supplement use in the United States has increased from 23% in NHANES I to 40% in NHANES III (7). For almost half a century in the United States, women have been prescribed prenatal vitamins containing folic acid during pregnancy. Therefore, free folic acid is unlikely to be a new phenomenon. We disagree that the findings of Morris et al support the theory that folic acid directly exacerbates the neurologic and neuropsychiatric effects of low vitamin B-12 status.

Even though 20% of the seniors in the study by Morris et al had serum folate concentrations >59 nmol/L, we think it is unlikely that such high serum folate concentrations are easily achievable from consuming folic acid–fortified food products anywhere consumption of RTE breakfast cereals is low. We found that the mean folic acid intake from folic acid fortification was 125 \(\mu g/d\) for all non-Hispanic white seniors; this value is similar to earlier estimates of the prevalence of dietary supplement use in the United States. Any person using a supplement containing folic acid should consider whether some underlying disease, such as undiagnosed pernicious anemia, might account for their findings. Any such study should be carefully evaluated so that appropriate inferences are made, especially when the findings are generalized to populations in whom the use of supplements and RTE breakfast cereals are low.

Solid evidence from randomized trials or cohort studies would be useful to better understand the relation between folic acid, vitamin B-12, and cognitive impairment. The apparently paradoxical findings of Morris et al warrant continued investigation.

We must continue to be vigilant in identifying potential adverse effects of folic acid fortification, but this effort must be founded on appropriate inferences. We must continue to examine the potential positive and negative effects of public health intervention programs and make decisions that will be safe for the entire population. Nevertheless, these decisions should not compromise the effectiveness of proven interventions, such as folic acid fortification, to prevent neural tube birth defects. Even if the findings of Morris et al are confirmed, we believe that it would be inappropriate to generalize their findings to countries in which the use of supplements and consumption of folic acid–fortified RTE breakfast cereals is low. For now, wheat flour for bread, which is fortified with folic acid, is likely the only potential source of synthetic folic acid elsewhere in the world. Based on Morris et al’s findings, the editorial by Smith cautions countries contemplating folic acid–fortification programs intended to prevent neural tube defects to delay such a decision. We believe that a delay in the implementation of folic acid–fortification programs elsewhere in the world on the basis of an inappropriate interpretation of Morris et al’s findings is not warranted and would be detrimental to public health.

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Reply to RJ Berry et al

Dear Sir:

Berry et al suggest that the results of our study (1) may apply only to American seniors and should not be generalized to countries considering fortification. Indeed, only American seniors were included in our study, and we sought to shed light on concerns expressed specifically for them. American seniors have been of particular concern not only because their vitamin B-12 status is low, but also because their consumption of fortified breakfast cereals and supplements is high (2). Consequently, American seniors are at high risk of exceeding the selected limit of <1 mg folic acid/d, including that in supplements, enriched cereal-grain products fortified under the government’s folate-fortification policy, and other fortified foods. Although we never suggested that our results had important implications for countries considering folic acid fortification, our findings are relevant to those countries to the extent that their populations resemble American seniors in folate-consumption habits, vitamin B-12 status, or both. The rate of supplement use in developing countries may be low, but dietary deficiency of vitamin B-12, which is found naturally only in animal products (3), is often high (4).

Berry et al are concerned that the comparison of subjects with very high serum folate concentrations with those with lower folate concentrations is equivalent to a comparison of supplement users with nonusers and that such comparisons could be confounded by differences between those groups not controlled by our multivariate adjustments. Because most of the characteristics Berry et al list correlate with good health, such residual confounding would not be expected to create associations between high folate status and adverse health consequences. To provide further assurance that the association between high folate status and cognitive impairment in seniors with low vitamin B-12 status was not caused by an unfair comparison, we restricted our analyses to supplement-using seniors with a low vitamin B-12 status. We found significant inverse continuous associations between serum folate and Digit-Symbol Coding scores in the 153 users of any dietary supplement (β coefficient = −0.09, P = 0.007) and in the 92 users of supplements that contained folic acid (β coefficient = −0.13, P = 0.003).

A related issue mentioned by Berry et al is their belief that our results, even if not spurious, have nothing to do with fortification. Although 32 of the 42 subjects with both a low vitamin B-12 status and a high serum folate concentration used supplements that contained folic acid, and another 8 reported eating a fortified breakfast cereal within 24 h of the interview, it is incorrect to conclude that fortification made no contribution to our results. Applying the same exclusion and inclusion rules as we applied in our study to data from the third National Health and Nutrition Examination Survey (NHANES III), we found that only 10% of 501 qualifying seniors who used supplements providing 400 μg folic acid had a serum folate concentration >59 nmol/L compared with 40% of 1999–2002 NHANES participants supplemented at the same level. Folic acid contributed by foods was calculated during the NHANES 2001–2002 survey, and the data showed that 100 μg folic acid from enriched cereal-grain products was often provided by single-meal portions of rice- or pasta-based dishes, pizza, tortilla-wrapped entrees, batter-fried chicken, hamburgers, bagels, muffins, and a variety of desserts. Folic acid from food boosted daily intakes of folic acid from enriched cereal-grain products to >100 μg for 40% of subjects with a serum folate concentration >59 nmol/L and low vitamin B-12 status. Cereal eaters typically obtained about another 100 μg/d from cereal, although some cereals were much more heavily fortified.

Berry et al wondered how low vitamin B-12 status and high serum folate coexisted in users of supplements that contained vitamin B-12. Although most of the supplements were conventional multivitamin-multiphaseeral combinations, it is difficult to know how much vitamin B-12 they provided. Such supplements almost always contained 400 μg folic acid, and the amount of vitamin B-12 attributed to survey participants from this source varied between 6 and 25–30 μg. However, an exact match for the reported product was found for only 18 of the 32 products recorded for the subjects with low vitamin B-12 status and high serum folate. Even 6 μg/d exceeds current recommendations of =3 μg/d (2), and normal aging should not affect absorption of crystalline vitamin B-12 (5). However, actual amounts and label-declared amounts may differ (6), and factors such as fasting status affect bioavailability (7). More importantly, the use of multivitamin formulations containing 2–30 μg vitamin B-12 have been found to be ineffective at normalizing vitamin B-12 status in community-dwelling elderly people (8). Furthermore, even a 6-wk course of 100 μg vitamin B-12d orally rarely normalized methylmalonic acid concentrations in a study of seniors attending a veterans’ affairs clinic who were identified through screening as having
low serum vitamin B-12 and elevated methylmalonic acid concentrations (9).

In asking whether the cognitive deficits of the cognitively impaired seniors with both low vitamin B-12 status and high serum folate could have been due to their low vitamin B-12 status, Berry et al seem to miss the point that the prevalence of cognitive impairment in this group exceeded that in the group with low vitamin B-12 status and lower serum folate (odds ratio: 2.6; 95% CI: 1.6, 6.6).

We never attributed the presence of unmetabolized folic acid in blood to folic acid fortification, as Berry et al claim, nor did we state that our findings support the theory that folic acid directly exacerbates the neurologic and neuropsychiatric effects of low vitamin B-12 status. In fact, we cautioned that our study could not address hypotheses specifically related to the effects of unmetabolized folic acid and noted consistencies and inconsistencies between our results and predictions of harm to seniors with a high folic acid intake. For example, contrary to expectations, we found more, not less, anemia in association with high serum folate when vitamin B-12 status was low.

Finally, we strongly agree with Berry et al’s support of continuing vigilance in examining potential positive and negative effects of public health intervention programs in an effort to make decisions that are safe for the entire population. In fact, this sentiment closely resembles that expressed by one of us in an editorial recently published in this Journal (10).

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Reply to RJ Berry et al

Dear Sir:

The information presented by Berry et al is of considerable interest, but perhaps not very surprising in that it shows that 92% of the elderly with the highest blood folate concentrations are consuming supplements containing folic acid. I did point out in my editorial that high blood folate concentrations, particularly unmetabolized folic acid, are not only a consequence of fortification but also of supplement use (1). What is irrefutable is that blood concentrations of folate increased dramatically in the United States after mandatory fortification was introduced, with the concentration of serum folate increasing from a median of 12.5 nmol/L to a median of 32.2 nmol/L (2). Furthermore, the skewness toward higher levels apparent before fortification is just as marked as after fortification (2); this skewness might be due in part to the use of supplements (3).

Whereas Berry et al focus on the elderly, these changes also relate to young children. The data reported by Pfeiffer et al (2) show that the section of the US population with the highest blood folate concentrations after fortification was children aged ≤5 y, 43% of whom had serum folate concentrations >45.3 nmol/L. Ten percent of these children had concentrations >77.3 nmol/L. Using the formula provided by Quinlivan and Gregory (4), we can estimate the dietary folate intake in folic acid equivalents: 43% of children aged <5 y are consuming the equivalent of >780 µg folic acid/d, ie, double the Institute of Medicine’s proposed tolerable upper limit (300–400 µg/d) for children of that age. What is of greater concern is that 10% are consuming the equivalent of >1320 µg folic acid/d, which is well above the tolerable upper limit of 1000 µg/d for adults. The next highest blood concentrations were found in children aged 6–11 y. It is plausible, as Berry et al suggest for the elderly, that such high concentrations are in part the result of supplement use in young children (5). However, it is perhaps more likely that the high concentrations in children are the consequence of 2 factors: the consumption of large amounts of bread and a diet rich in fortified ready-to-eat breakfast cereals. We simply do not know whether these high blood concentrations cause harm, but it must be of concern that such concentrations occur in children during a rapid stage of development. The study by Morris et al (6) highlights the potential importance of the correct balance between folate and vitamin B-12 in the elderly and we should consider whether a similar balance is important in young children. We should also consider whether the similar balance is important in young children, especially in parts of the world where many children have a low vitamin B-12 status.

Berry et al believe that a delay in implementing folic acid fortification in other countries would be detrimental to public health. This is likely to be the case in relation to neural tube defects, but should millions of people have to eat food fortified with folic acid without choice? The point that I was trying to make in my editorial is that fortification might potentially harm more people than it would benefit. The benefit of fortification in relation to neural tube defects
would be negated if only 1 in 100,000 subjects eating folic acid–fortified food in the United Kingdom and North America experienced a serious adverse effect. Mandatory fortification exposes the entire population to extra folic acid, including those that might be susceptible to harm from such. This situation is quite different from the individual choice of taking supplements and from targeted interventions that might in the future be indicated for some sectors of the population (7) in addition to women planning to become pregnant. What we can all agree on is that more research is needed concerning the benefit and harm of folic acid. In my opinion, it is the responsibility of health authorities to conduct such research before folic acid fortification is introduced in additional countries on the basis of the evidence that is currently available.

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Over the past 20 y, spectacular advances in the molecular biology of selenium have far outstripped growth in knowledge of its role in nutrition and health. Although many novel details of selenoprotein synthesis have been discovered and selenoproteomes of a number of species have been characterized, most hypotheses on physiologic and health-related selenium activities still lack experimental support and remain speculative in nature. Enter this book by Peter Surai, which has the stated goal of providing up-to-date information about the role of selenium in the nutrition and health of humans and farm animals.

This is a very long book; it contains 14 chapters that range in length from 32 to 168 pages. Topics covered clearly reflect the author’s interests in antioxidant protection and animal science, although the longest chapter of the book is on human health. Surai writes in an earnest style with frequent declarations in the form of, “Did you know that...?” These stand-alone statements are clearly intended to impart important facts and sometimes achieve their goal. References are numerous and contain titles, which makes them useful.

The opening chapter addresses antioxidant systems, and it introduces the theme of oxidative stress that runs through the book. The discussion of these systems, however, is confined to the level of reactive molecular species and enzymes that interact with them. Important current topics in the area of oxidative stress, such as oxidant- and antioxidant-related signaling systems and transcription factors, are not covered. Thus, this chapter has the feel of having been written a decade or more ago and represents one author’s synthesis of how organisms use antioxidant enzymes.

Some of the length of the chapter on selenium and human health is due to its discussion of nonselenium aspects of the diet. Moreover, although the chapter makes some important points, not enough effort is made to assess the significance of most of the works presented. This leads to a listing of articles that support the involvement of selenium in various health conditions and to statements such as, “Clearly, Se appears as the key micronutrient in prevention of cancer, cardiovascular, inflammatory and infectious diseases.” Such a statement does not reflect a useful assessment of the available data.

A particularly troubling feature of this book is its touting of a specific commercial product, a yeast form of selenium that is sold to provide selenium to animals. The book notes that selenomethionine is the major form of selenium in plants (and yeast) and that feeding selenomethionine raises the tissue selenium concentrations in animals more than does feeding inorganic forms of selenium. Surai devotes a chapter to selenium-enriched eggs, milk, and meat produced in this way. He argues that enriching the food chain in this way will provide selenium to people who presumably need it. Unfortunately, adoption of this approach might increase intake of animal products when a comparable increase in selenium intake could be achieved by other means. Moreover, a need for selenium supplementation should be established before such procedures are adopted.

In conclusion, this book does not succeed in providing a concise, critical update on selenium in nutrition and human health. It is overly long, contains a great deal of extraneous material, and does not adopt a critical stance. Finally, the book’s promotion of a commercial product casts doubt on its objectivity.

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